CLATHRIN-MEDIATED ENDOCYTOSIS OF YOLK PROTEIN IN THE *DROSOPHILA* OOCYTE

by

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The developing egg chamber of *Drosophila* accumulates yolk protein to utilize during embryonic development. Egg chamber development is divided in 14 different stages, yolk protein endocytosis occurs between stages 8-11. Yolk protein is present in hemolymph, and the ovaries are bathed in hemolymph. Yolk protein forms a complex with Yolkless for endocytosis in oocyte. Yolkless is an LDL receptor superfamily member, and its cytosolic tail contains the signal for internalization by clathrin-mediated endocytosis. The Yolkless cytosolic tail harbors an atypical FxNPxA sequence instead of the commonly found tyrosine-based sorting signal FxNPxY. The tyrosine-based sorting signals interact with PTB domain-containing proteins. In the present study, I show that PTB domain-containing phagocytic protein Ced-6 is expressed in the cortical region of oocyte of the egg chamber. Ced-6 harbors all the biochemical properties of clathrin-associated sorting proteins. Ced-6 can physically interact with clathrin, AP-2 and phosphoinositide(4,5)P_2. The Ced-6 PTB domain interacts with the cytosolic tail of Yolkless and clusters Yolkless into clathrin-coated pit. This clustering leads to internalization of Yolkless by clathrin-mediated endocytosis. In conjunction with the FxNPxA signal, Yolkless cytosolic tail contains a functional, although atypical, dileucine signal. This atypical dileucine signal can physically interact with AP-2. This interaction leads to the clathrin-mediated endocytosis of Yolkless. Clathrin adaptor AP-2 and Ced-6 function redundantly in yolk

protein uptake, as Ced-6-null flies accumulate yolk protein. Results from this study led to a model where both AP-2 and Ced-6 interact with Yolkless independently to ensure its internalization. Interaction of Ced-6 with clathrin and AP-2 leads to effective clustering of Yolkless for internalization by clathrin-mediated endocytosis.

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PREFACE

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I dedicate this work to all the STROKE SURVIVORS (strokies).

ABBREVIATIONS

AP-2	Adaptor protein-2
APP	Amyloid precursor protein
ARH	Autosomal recessive hypercholesterolemia
ATP	Adenosine triphosphate
BAR	BIN-amphiphysin-RVS
BSA	Bovine serum albumin
CCV	Clathrin-coated vesicle
CHC	Clathrin heavy chain
CLASP	Clathrin associated sorting adaptor
CLC	Clathrin light chain
CME	Clathrin-mediated endocytosis
CP	Coated pit
CV	Coated vesicle
Dab	Disabled
DIAG	Diacyl glycerol
Diap1	Diaphanous homolog 1
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EGFD	EGF precursor domain
EGFR	Epidermal growth factor receptor
EST	Expressed sequence tag
FBE	Fat body enhancer

FBS	Fetal bovine serum
GFP	Green fluorescent protein
GST	Glutathione S-transferase
GTP	Guansine triphosphate
GULP	enGulfment adaptor protein
HRP	Horse radish peroxidase
HSC	Heat shock cognate
ITAM	Immunoreceptor tyrosine activation motif
LAT	Linker of activated T cells
LBD	Ligand binding domain
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
NAK	Numb-associated kinase
OCRL	Oculocerebrorenal syndrome of Lowe
OE	Ovarian enhancer
OLSD	O-linked sugar domain
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PH domain	Pleckstrin homology domain
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PTB	Phosphotyrosine binding
PtdIns(4,5)P_2	Phosphatidlyinositol(4,5) bisphosphate
mCRFP	Monomeric Cherry Red fluorescent protein

RAP	Receptor associated protein
RNA	Ribonucleic acid
RNAi	RNA interference
siRNA	Small interfering RNA
SNX9	Sorting nexin 9
Tf	Transferrin
TGN	Trans-Golgi network
TMD	Transmembrane domain
VgR	Vitellogenin receptor
VLDLR	Very low-density lipoprotein receptor
WASP	Wisskott-Aldrich syndrome protein
YP	Yolk protein
YPF	Yolk protein factor

1.0 Introduction

Reading a mechanistic understanding of the process of endocytosis of yolk proteins in the *Drosophila* oocyte is the major focus of this thesis. For my thesis work, I have used *Drosophila* ovaries as a tool to further understand clathrin-mediated endocytosis. The development of the ovary in a mature *Drosophila* female is a cyclical process and is highly dependent on nutritional status, which can influence yolk protein endocytosis. Profoundly I have divided the Introduction in two major parts: in the first, I briefly discuss endocytosis and its different routes. I also describe the known characteristics of the proteins further studied in this dissertation and their known role in clathrin-mediated endocytosis. In the second part of the Introduction, the main events of *Drosophila* oogenesis are discussed. The principle outcome of this dissertation is a more coherent and complete understanding of clathrin-mediated endocytosis (CME) during the accumulation of yolk protein in *Drosophila*.

1.1 Endocytosis

The plasma membrane creates a surface through which a cell can communicate with its environment. Proper communication not only depends on extracellular stimuli but also on a tight regulation of plasma membrane constituents. Bidirectional flux of membrane-coated vesicles is one of the important processes that maintain a correct membrane composition. In the process termed endocytosis, membrane-bound vesicles have an inward cytosolic movement.

Endocytosis at the plasma membrane can occur through various routes. Different vesicular structures are detected at the plasma membrane. The pits decorated with clathrin

show an electron-dense coat and can be easily detected with electron microscopy; non-clathrin-coated pits that contain caveolin are also observed at the plasma membrane (Hansen and Nichols, 2009). Endocytosis can also be initiated by macropinocytosis, which generally take place in highly ruffled regions of plasma membrane. In addition to vesicular structures, some tubular structures are observed in the plasma membrane, and these structures may also become a part of budding vesicles that initiate endocytosis (Marbet *et al.*, 2006). One of the endocytic pathways that occur in specialized cells is called phagocytosis. This process is involved in uptake of large membrane areas and extracellular objects. Endocytic pathways depend on particular lipids, membrane curvature and cytoskeletal elements, and disruption of endocytosis may lead to certain diseases like autosomal recessive hypercholesterolemia (ARH), myopathies, muscular dystrophy, neurodegeneration, cancer and certain immune diseases (Doherty and McMahon, 2009).

1.1.1 Phagocytosis

The results of this study lead to some speculation on the possible role of clathrin-mediated endocytosis in phagocytosis. Therefore, I will briefly discuss the main events of phagocytosis. Phagocytosis is a key component of the innate and adaptive immune responses. It is now accepted that phagocytosis plays an important role in development and tissue homeostasis. Particles larger than 0.5 μm are internalized through phagocytosis (Rejman et al., 2004). Phagocytosis is initiated by receptors present at the plasma membrane. Macrophages, dendritic cells and neutrophils are professional phagocytes that contribute to the first line of defense against bacterial and fungal infection. Endothelial

cells, epithelial cells and fibroblasts can also perform phagocytic activity. They engulf the apoptotic cells and help clear dead cells (Rabinovitch, 1995).

Phagocytosis is a complex process, and different molecular mechanisms exist. For example, phagocytosis engulfing a bacterium or an apoptotic cell can initiate different pathways. Particles of a wide variety are engulfed by phagocytosis, and therefore numerous different receptors can initiate this process. The best understood molecular mechanism of phagocytosis is that mediated by leukocyte FCγ receptor (FcγR) activity. The FcγR binds to Immunoglobulin G (IgG)-opsonized particles and directs internalization of them. The cytoplasmic domain of the FcγR is altered due to the binding of IgG-opsonized particles and recruits several signaling proteins, which reorganize actin and play an important role in the extension of membrane around the particle to be engulfed (Cox and Greenberg, 2001). The phagocytic cup is sealed to form the phagosome, and the phagosome then fuses with a sorting endosome (Vieira *et al.*, 2002). Data from several studies suggest that phagosome maturation follows the same path as that of predefined endocytic compartments. Therefore, these phagosomes fuse with early endosomes, late endosomes and lysosomes in sequential manner (Vieira *et al.*, 2002).

Different receptors are required to initiate phagocytosis, and not all potential phagocytic receptors are necessary during the ingestion of a particular particle (Griffin *et al.*, 1975a; Griffin *et al.*, 1975b). During pseudopod extension and engulfment, some contents of the plasma membrane that are not required for phagocytic cup formation are removed, perhaps by clathrin-mediated endocytosis (Swanson, 2008). An electron microscopy (EM) study revealed the presence of clathrin-coated buds on the phagocytic cups (Aggeler and Werb, 1982). Moreover, the depletion of clathrin in S2 cells of

Drosophila inhibits the phagocytosis of *E. coli* (Rocha *et al.*, 2011). These data hint at a link between clathrin-mediated endocytosis and phagocytosis.

1.1.2 Clathrin-mediated endocytosis

Electron microscopic images of mosquito oocytes taken by Roth and Porter (1964) revealed a proteinaceous coat around an invagination of the plasma membrane. Clathrin is the main component of the proteinaceous coat (Pearse, 1975). Highly coordinated clathrin-coat formation occurs when cargo-bearing receptors are recognized by clathrin-associated proteins. The progression of clathrin-mediated endocytosis is divided into different stages: initiation, cargo selection, coat formation, scission and uncoating (Figure 1.1).

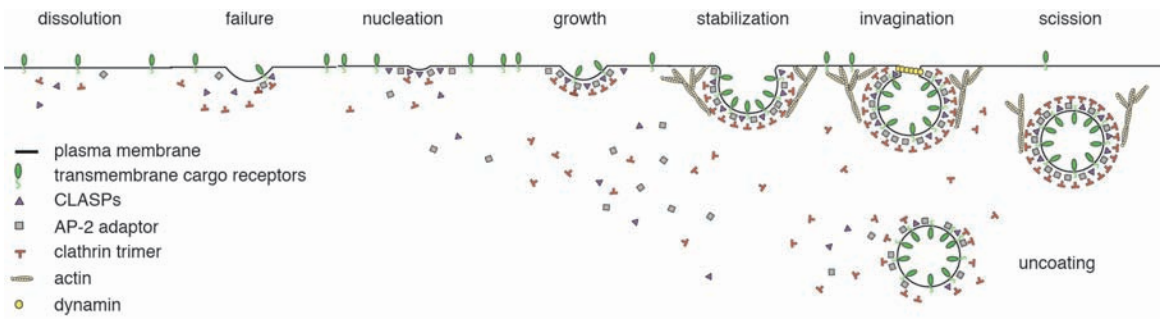


Figure 1.1: Clathrin-mediated endocytosis

Clathrin-coat assembly involving Nucleation, Growth, stabilization, invagination, scission leading to uncoating. Adapted From Traub (2009).

Clathrin and other necessary or accessory proteins are cytosolic and are recruited to the plasma membrane, and these proteins are reused after budding of vesicles from plasma membrane and uncoating of the vesicles. Clathrin-mediated endocytosis internalizes various receptors including the epidermal growth factor receptor (EGFR), the low density lipoprotein receptor (LDLR) and the transferrin receptor. Endocytosis of EGFR is stimulated by its ligand; the LDLR and transferrin receptor are internalized constitutively. Generally these various types of transmembrane receptors and their cargo are concentrated at clathrin-coated pits and can carry a wide range of sorting signals at their cytoplasmic tails. The sorting signals, which are recognized by different clathrin-associated sorting proteins, are discussed later.

Initiation of clathrin-coat formation is a very complex process, which involves the plasma membrane enriched lipid phosphoinositide(4,5)-bisphosphate (PIP_2) and clathrin-coat proteins. Adaptor protein-2 (AP-2) is the principal non-clathrin protein of the clathrin coat (Blondeau *et al.*, 2004). The clathrin coat forms a cage like structure that, with the help of accessory proteins, can stabilize membrane deformation as the membrane transitions from a shallow structure to a highly invaginated bud (Hinrichsen *et al.*, 2006; Traub, 2009). Several BAR (Bin-Amphiphysin-Rvs) domain proteins are recruited during the stages of clathrin bud formation (Taylor et al., 2011). These BAR domain proteins can bind to the membrane and have preferences for different curvatures of membrane (Peter et al., 2003). These BAR domain-containing proteins also recruit a mechanochemical enzyme, dynamin (Ferguson et al., 2009; Kosaka and Ikeda, 1983). Dynamin is polymerized into a ring at the neck region of a coated bud; upon GTP hydrolysis by dynamin, the mature bud is severed from the plasma membrane (Hinshaw and Schmid, 1995; Sweitzer and Hinshaw, 1998). After the fission from the plasma membrane,

clathrin is rapidly disassembled from the vesicle in an ATP-dependent process that relies on the heat shock cognate 70 (Hsc70) chaperone and its cofactor auxilin (Schlossman *et al.*, 1984; Ungewickell *et al.*, 1995). This uncoated vesicle matures or fuses with the endosome. The phosphoinositide composition of the vesicle changes as it progresses from plasma membrane to the endosome. The phosphatases synaptojanin, Ship-2 and OCRL1 might be required to alter the composition of phosphoinositide (Nakatsu *et al.*, 2010) during the uncoating.

1.1.2.1 Sorting signals in clathrin-mediated endocytosis

Discrete sorting signals present within different transmembrane cargo receptors avoid competition between clathrin-mediated cargo and other endocytic cargo. Moreover, these sorting signals allow increases in the concentration of specific cargo in the designated pit and eventual preferential budding from membrane. The cytosolic domain of transmembrane receptor cargo can carry a linear peptide sequence as a signal or molecules like ubiquitin that are added post-translationally.

The sorting signals in clathrin-mediated endocytosis are mainly tyrosine-containing motifs such as YxxΦ or NPxY (Chen *et al.*, 1990; Ohno *et al.*, 1995b). However, other types of sorting signals, like dileucine motifs (Marks *et al.*, 1996), are also found. These types of signals are generally present in the transmembrane receptor cargos that are internalized constitutively. By contrast, several transmembrane receptor cargoes are only selectively internalized after an extracellular stimulus is received. Certain post-translational modifications like ubiquitin also act as sorting signals (Hawryluk *et al.*, 2006; Hicke and Riezman, 1996). Another post-translation modification, phosphorylation, may enhance the internalization of cargo (Lundh *et al.*,

2009; Traub, 2009). Certain transmembrane receptor cargos utilize multiple sorting signals for rapid internalization (Li *et al.*, 2001), whereas some types of cargo utilize different sorting signals depending on the fate after internalization (Traub, 2009). Sometimes multiple signals are read in a hierarchical way for the internalization by clathrin-mediated endocytosis (Traub, 2009). Thus, a diverse set of internalization sorting signals have evolved to allow differential regulation and plasticity in clathrin-mediated endocytosis.

1.1.2.2 Clathrin

As my study is focused on clathrin-mediated endocytosis, and as I have tried to biochemically dissect binding sites for clathrin, I will discuss briefly the known properties of clathrin and the interaction motifs present in endocytic proteins for clathrin binding. Clathrin is the major coat protein in purified clathrin-coated vesicles. In solution, clathrin exists as a heterohexamer of three heavy and three light chains assembled in a structure called a triskelion (Kirchhausen, 2000). Purified clathrin can self assemble into cages, but *in vivo* its assembly is regulated by interactions with several proteins. The clathrin heavy chain of a mammal is ~1675 residues long; the carboxy-terminal sequence depends on the species (Brodsky *et al.*, 2001). The C-terminus of clathrin heavy chain is responsible for the formation of triskelion through trimerization. This region also binds to the clathrin light chain. The N-terminal domain of the clathrin heavy chain contains the globular terminal domain. This terminal domain forms a seven-bladed β-propeller structure. This domain contains binding sites for various clathrin binding proteins (Fotin *et al.*, 2004; ter Haar *et al.*, 1998); so far, four interaction sites for these proteins have been detected in the N-terminal domain (Willox and Royle, 2011).

Initially, a short consensus sequence was identified in some clathrin-associated proteins that is sufficient to bind to the N-terminal domain of clathrin (Krupnick et al., 1997) (Dell'Angelica, 2001; Dell'Angelica et al., 1998; Ramjaun and McPherson, 1998). This sequence, L[L/I][D/E/N][L/F][D/E], is known as the clathrin-box motif. Sequence alignment and structural studies indicate that the cognate binding site is located in a groove between blades 1 and 2 of the clathrin terminal domain (Goodman *et al.*, 1997); the critical features of clathrin box motif are LΦPΦ, where L is leucine, Φ denotes a bulky hydrophobic acidic residue and, P is a polar residue (ter Haar *et al.*, 2000). Some clathrin-binding proteins do not have a clathrin box sequence, instead these proteins have a type II clathrin box containing sequences such as PWDLW and LMDLA (Drake and Traub, 2001). In addition, a splice variant of arrestin 2 utilizes an [LI][LI]GXL sequence to bind to the clathrin terminal domain at a site between blades 4 and 5 (Kang *et al.*, 2009). Recently it was found that the end of blade 7 and the region connecting blade 7 to blade 1 of clathrin heavy chains is also important for its interaction with several proteins (Willox and Royle, 2011).

A potential clathrin box sequence found in mammalian AP180 is in inaccessible region of the folded protein (Mao *et al.*, 2001). This suggests that not all clathrin box sequences are functional even in an endocytic protein. There is yet another version of the clathrin box motif designated as DLL or SLL. This sequence is found in mammalian AP180 and hetero-tetrameric adaptor proteins (Mao *et al.*, 2001; Morgan *et al.*, 2000). These unstructured regions contain highly repetitive sequences necessary for clathrin binding (Lafer, 2002). Clathrin terminal domain interacting motifs are often present in several copies and the multiple clathrin binding sites in any endocytic protein minimize its dissociation from clathrin. Each clathrin triskelion contains three terminal clathrin

domains and therefore the terminal domain can bind to more than one clathrin binding protein; this may increase retention of the clathrin triskelion at endocytic sites to promote rapid coat assembly (Zhuo *et al.*, 2010).

1.1.2.3 Adaptor protein complex

There are five known heterotetrameric adaptor protein complexes (designated as AP-1, AP-2, AP-3, AP-4 and AP-5) (Boehm and Bonifacino, 2001; Hirst et al., 2011), which can function in different pathways in membrane trafficking. The AP-1, AP-2 and AP-3 complexes have been shown to interact with clathrin. In clathrin-coated vesicles, the inner side of the coat contain AP-1, AP-2 or AP-3. Loss-of-function or gain-of-function assays have been used to deduce the role of these AP complexes. AP-2 is present exclusively at plasma membrane and is the main protein found on purified endocytic clathrin-coated vesicles (Traub, 2009). AP-1 functions in protein sorting in the late Golgi/trans-Golgi network (TGN) or endosomes. AP-3 might have a function in sorting the proteins between TGN and lysosomes (Simpson *et al.*, 1997). These three complexes recognize the tyrosine-based Yxxϕ or dileucine-based sorting signals present at the cytosolic tail of transmembrane receptors (Traub, 2009).

Each AP complex consists of two large polypeptide chains (one each of γ/α/δ/ε/ζ and β1-5 respectively), one medium size chain (μ1-5) and one small chain (σ1-5) (Hirst *et al.*, 2011; Robinson and Bonifacino, 2001). The subunits of AP complexes are also sometimes known as adaptins. These adaptins are transcribed from separate genes and present as one or more closely related isoforms. In *Drosophila*, the β-subunit is shared by both AP-1 and AP-2 complexes (Camidge and Pearse, 1994). *Drosophila melanogaster, Caenorhabditis elegans* and *Saccharomyces cerevisiae* contain only three AP complexes

instead of the five known AP complexes present in mammals (Boehm and Bonifacino, 2001).

Since the AP-2 complex is expressed at the plasma membrane and is involved in clathrin-coated pit formation for endocytosis, I will briefly discuss the structure of AP-2. I will also mention the interaction motif/signals present in endocytic proteins necessary for the binding of AP-2.

1.1.2.3.1 Adaptor protein-2

Each large subunit of AP-2 can be divided into the folded trunk domain and the folded appendage domain. The trunk and appendage domains of the large subunit are connected by a proteolytically sensitive flexible linker (Kirchhausen *et al.*, 1989; Zaremba and Keen, 1985). Four subunits (α, β2, μ2 and σ2) without the appendage domains make up a biochemically stable core (Collins et al., 2002b). The appendage domain of the large subunits contain a clathrin binding domain, the membrane targeting information and the platform subdomain needed for accessory protein recruitment (Reider and Wendland, 2011) (Figure 1.2).

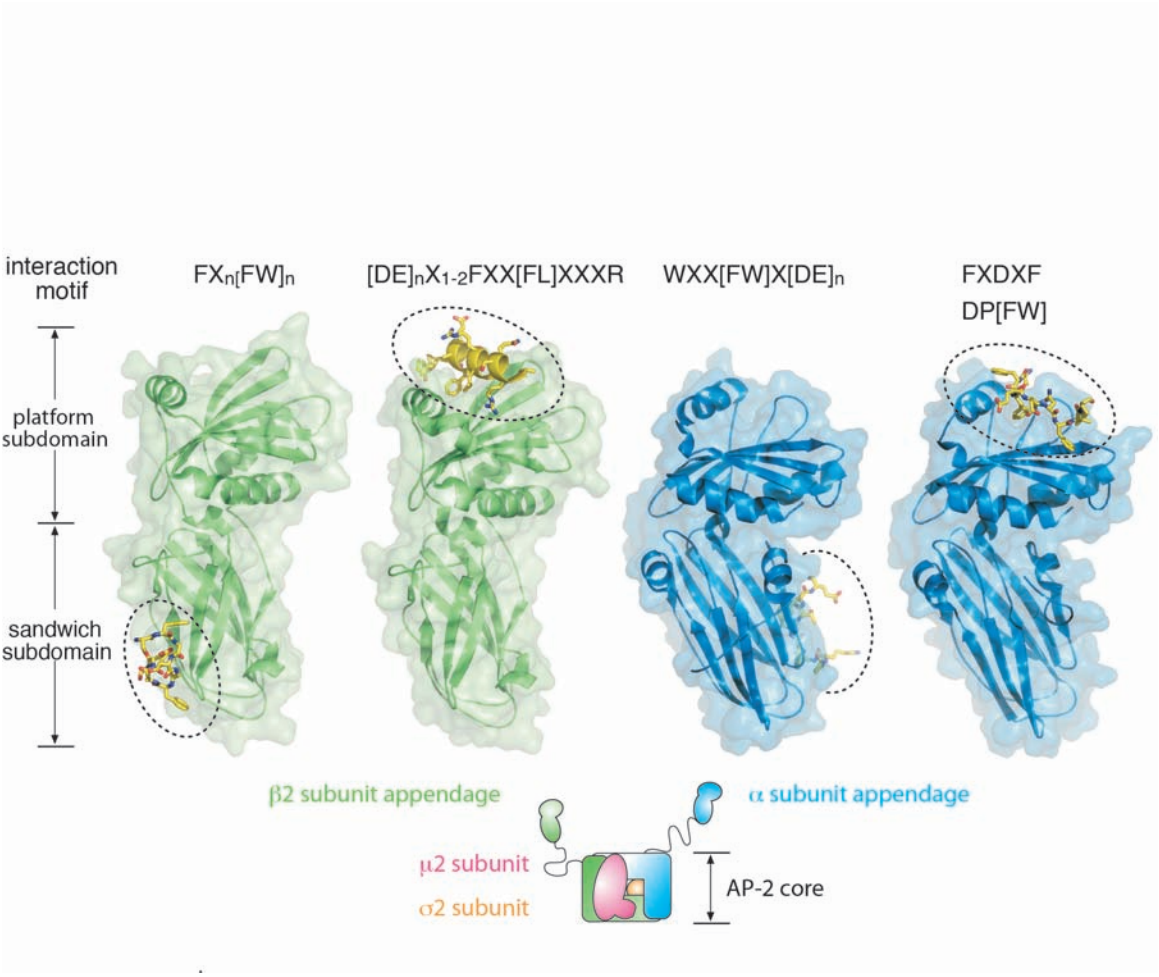


Figure 1.2: Illustration of appendage interaction surface

α subunit and β2 subunit of AP-2 can be divided in platform and sandwich domain which can interact with different motifs present in endocytic proteins. Adapted from Traub (2009).

Both appendages of the large subunits of AP-2 are structurally similar and have analogous bilobal structures. The N-terminal regions of both forms a sandwich β subdomain, while the C-terminus forms the platform subdomain (Edeling *et al.*, 2006b). The platform subdomain of the α appendage engages DPW/DPF interaction motifs (Brett *et al.*, 2002), and the sandwich subdomain of the α appendage interacts with the WxxF

motif (Jha *et al.*, 2004; Praefcke *et al.*, 2004; Ritter *et al.*, 2004). The DPW/DPF motif can also interact with platform subdomain of the β appendage because of its structural similarity with the α appendage domain. However, the β appendage platform subdomain was shown to selectively bind to a 16-amino acid sequence from ARH and β-arrestin (Edeling *et al.*, 2006a; Mishra *et al.*, 2005), and this 16-residue peptide has a much higher binding affinity for this sequence than does the DPF/DPW motif (Edeling *et al.*, 2006a). Thus, AP-2 not only acts as bridge between clathrin and membrane but also interact with several clathrin-associated sorting proteins through its appendage domain.

The medium μ2 subunit of AP-2 interacts with the Yxxϕ type sorting signal (Ohno *et al.*, 1995a; Owen and Evans, 1998). The AP-2 complex also recognizes the [DE]XXXL[L/I]-type dileucine signal. Each sorting signal is recognized by a different subunit of AP-2, thus avoiding competition for recognition of receptors (Marks *et al.*, 1996). The σ2 subunit of AP-2 is responsible for recognizing dileucine sorting signals and also forms a hemicomplex with the α subunit that is required for stabilization of the interaction (Doray *et al.*, 2007; Traub, 2009).

Phosphorylation and phosphoinositide binding regulate AP-2 function (Huang *et al.*, 2003; Olusanya *et al.*, 2001; Ricotta *et al.*, 2002; Semerdjieva *et al.*, 2008). Even though AP-2 is the main constituent of clathrin-coated vesicles and sorts several cargoes, depletion of ≥ 90% of the AP-2 does not affect the internalization of certain cargo such as LDLR (Motley et al., 2003). Therefore, adaptors in addition to AP-2 must function in clathrin-mediated internalization. It is now accepted that various monomeric adaptors designated as clathrin-associated sorting protein (CLASPs) are required to sort certain cargo.

1.2 Phosphotyrosine-binding (PTB) domain

PTB domain-containing proteins are required for clathrin-mediated endocytosis of certain types of cargo, like the LDLR and integrins (Morris and Cooper, 2001; Teckchandani et al., 2009). The PTB domain was first characterized in 1994 (Kavanaugh and Williams, 1994) as a protein domain that recognizes a phosphotyrosine specific peptide. The PTB domain protein are members of PH superfold family and PH domains are typical examples of modular phosphoinositide binding domains. Therefore all PTB domains share this membrane binding feature, and they are localized to the juxtamembrane region to interact easily with receptors (Uhlik et al., 2005). Most PTB domain-containing proteins can be segregated into those that require phosphorylation of ligand and those that do not. However, there are some exceptions, including SNT1 (also known as FRS2) that can bind to both phosphorylated and non-phosphorylated ligands (Dhalluin *et al.*, 2000; Ong *et al.*, 2000; Yan *et al.*, 2002).

1.2.1 Basic structure of PTB domain

High-resolution studies of a PTB domain-containing proteins revealed the folding features of the PTB domain (Zhou *et al.*, 1995). All PTB domains have a basically similar 3D structure regardless of the primary sequence, and all have a PH-domain superfold (Uhlik et al., 2005). The general mode of ligand recognition is shared by all PTB domain-containing proteins. The ligand (a peptide) generally forms a pseudo-β sheet that contacts the β5 strand and the α helix of PTB domain (Uhlik *et al.* 2005). The peptide ligand also forms a type 1 β turn, and this twirl is formed by the consensus Asn-Pro-X-Tyr (or NPxY) motif (Hutchinson and Thornton, 1994). The Tyr in the NPxY sequence is designated as the 0 position and amino acid residues amino terminal are

referred to as "-" and amino acids residues carboxy terminal are referred to as "+" relative to Tyr0 (thus, the Asn in the sequence is referred as Asn -3). However, both subtle and extreme variations of the NPxY motif do exist. To accommodate this, depending on the protein, additional helices and strands provide additional structural regions that permit recognition of distinct ligands (Farooq and Zhou, 2004). Several studies have found that [FY]XNPx[YF] sorting signals are recognized by a PTB domain containing subfamily of CLASPs (Traub, 2009).

Only a few studies have evaluated the gene regulation of PTB domain-containing proteins. In *Xenopus* oocytes, XARH is an adaptor protein required for the uptake of lipoprotein (Zhou *et al.*, 2003). Two isoforms of *Xenopus* ARH exist. These two transcripts have different 3'UTRs and the large transcript is localized to the vegetal cortex of *Xenopus* oocytes (Zhou *et al.*, 2004). We have described a mosquito ARH designated trephin; Expression of trephin in the oocyte is induced in a manner depending on the nutritional status of mosquito oocytes (Figure 1.3) (Mishra *et al.*, 2008). This transcriptional regulation of trephin might be required to enhance the internalization of vitellogenin after a blood meal. As in *Xenopus* oocytes, the vitellogenin receptor of mosquito is present at very high concentrations, therefore the elevation in internalization rate depends on the expression of adaptor proteins (Shankaran et al., 2007).

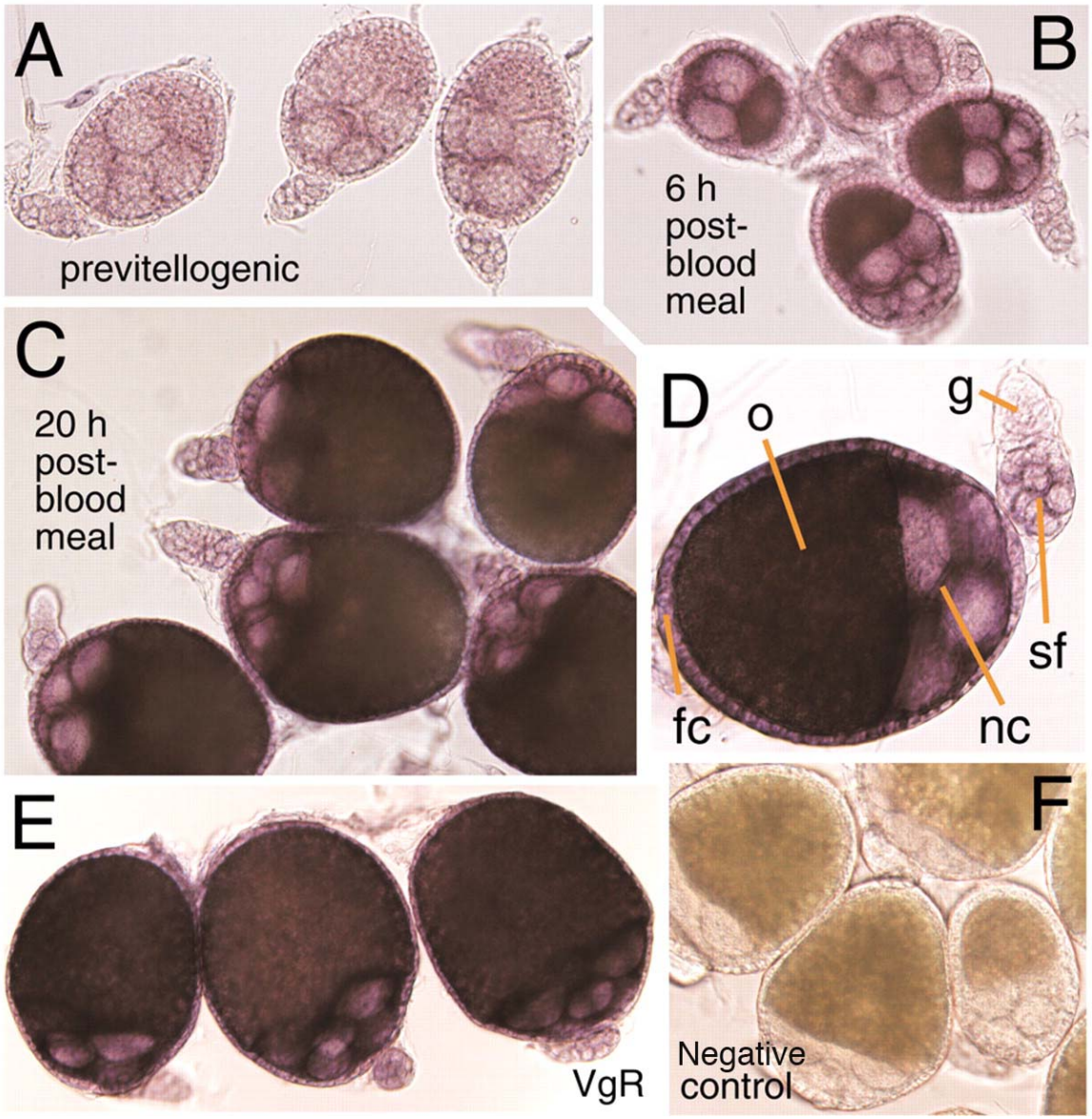


Figure 1.3: Upregulation of trephin transcripts during vitellogenesis in *Aedes aegypti.*

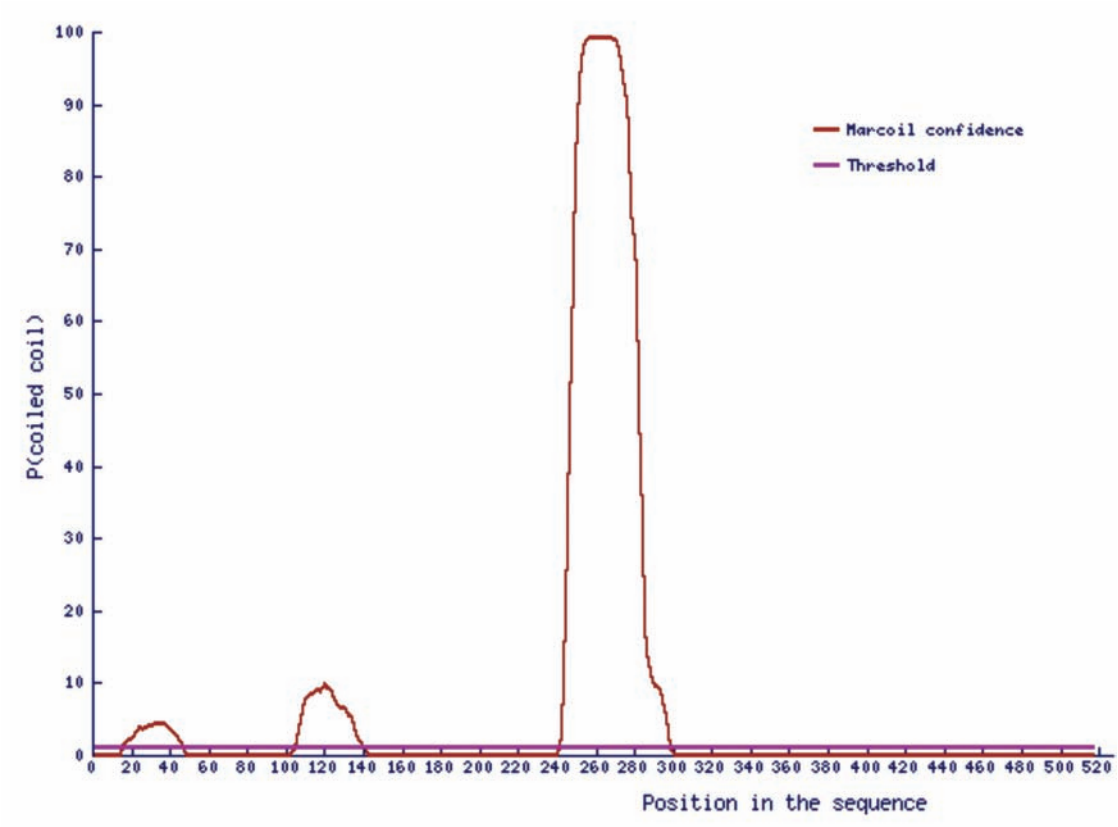
Whole-mount *in situ* hybridization analysis of *Aedes* female ovaries harvested from previtellogenic (A), 6 hours after the blood meal (B) or 20 hour after the blood meal (C-F). Paraformaldehyde fixed ovaries were hybridized with antisense trephin (A-D), *Aedes* vitellogenin receptor (VgR) riboprobes with (E) or without (F) riboprobe. Purple staining indicates the positioning of transcripts. Fc, follicle cells; g, germarium; nc, nurse cells; o, oocyte; sf, secondary follicle (taken from the published work of **Mishra and Jha** *et al.*, 2008, with permission).

1.3 Ced-6/Gulp

Ced-6 is also a PTB domain containing protein that interacts with the NPxY signal present in the cytosolic tail of certain receptors. A genetic screen in *C. elegans* (Hengartner, 1997) to study cell engulfment within the apoptotic pathway revealed six relevant genes. CED-6 was encoded by one of those six genes, and thus the Ced-6 protein was first characterized in *C. elegans*. At the N-terminus of the CED-6 protein is a PTB domain, and at the C-terminus there are several proline/serine-rich regions that contain the PxxP sequence (Liu and Hengartner, 1998). A stretch of hydrophobic and highly charged amino acids between the N-terminus and C-terminus are responsible for dimerization of the *C. elegans* CED-6 as well as Ced-6 homologs from different species (Figure 1.4).

CED-6 functions downstream to CED-1 and CED-7. CED-1 encodes a receptor that contains NPxY and YxxL sorting motifs, and the receptor is essential for engulfment of cell corpses (Zhou *et al.*, 2001). The CED-1, CED-6 and CED-7 pathway along with CED-2/CrkII, CED-5/Dock180 pathway are required for actin reorganization during the engulfment of apoptotic cells in *C. elegans* (Kinchen *et al.*, 2005). Studies done in *Drosophila* glial cells revealed a CED-1 ortholog called Draper (Freeman *et al.*, 2003), which was found to be essential for clearance of severed axons cellular debris by glial cells (MacDonald *et al.*, 2006). The cytosolic tail of Draper contains an NPxY motif, which interacts with the PTB domain of *Drosophila* Ced-6 (Awasaki *et al.*, 2006). The mammalian homolog of the Ced-6 protein is known as Gulp (enGulfment adaptor protein) (Su *et al.*, 2002). *Drosophila* Ced-6 contains 62 extra amino acids at N-terminus and 151 extra amino acids at C-terminus relative to the human protein.

Predicted coiled-coil domain of *Drosophila* Ced-6



http://coiledcoils.chm.bris.ac.uk/scorer/Dm-Ced-6

Amino acid alignment of predicted dimerization domain

```
GULP1_Hs      160  -VETRKQIAGLQKRIQDLETENMELKNKVQDLENQLRITQVS-------- 200
CED6_Ce       199  -LENQKQIYILKKKIVELETENQVLIERLAEALRANSKADYENTGPPIYP 247
CED6_Dm       249  LSKAQQQINHLQQTVNVYKERLREVSAKLPKAELDALLFNLGIKDILEAP 298
```

Figure 1.4: Predicted coiled-coil region of Ced-6.

Predicted coiled-coil region of *Drosophila* Ced-6 (top panel), X-axis denotes the number of amino acids of the *Drosophila* Ced-6 sequence and Y-axis is the threshold of predicted coiled-coil region. Amino acid sequence alignment (bottom panel) of *Dm* Ced-6 (Uniprot: Q7JUY7) compared to *C. elegans* Ced-6 (Uniprot: O76337) and human Gulp1 (Uniprot: Q9UBP9) with conserved residues in pink and the heptad repeats with proper positioning of hydrophobic and charged residues.

The PTB domain of Ced-6 is highly conserved across species. The NPxY motif from *C. elegans* CED-1 is efficiently co-precipitated with GULP (Su *et al.*, 2002). That same study also demonstrated that the NPxY sequence of CD91/LRP can interact with the PTB domain of GULP, while GULP failed to interact with the NPxY sequence of the β3-integrin chains (Su *et al.*, 2002). Stabilin-2/FEEL-2/HARE is a large glycoprotein that functions as a scavenger receptor and can also bind to bacteria (Adachi and Tsujimoto, 2002; Baba *et al.*, 2001). Stabilin is required for engulfment of apoptotic cells (Park *et al.*, 2008). The cytoplasmic tail of stabilin-2 contains the NPxY motif, which is required for its interaction with GULP (Park *et al.*, 2008). All of these studies have shown that Ced-6 and its orthologs in different organisms interact with engulfing receptors and help in phagocytosis.

Recently, Ced-6 has been also found in non-phagocytic cells. The rat homolog of Ced-6 has been detected in neurons, and sub-cellular fractionation experiments suggest that it is absent from phagocytic glial cells (Martins-Silva *et al.*, 2006). *Drosophila* Ced-6 was found in dying cells and is essential for autophagy in the salivary gland (McPhee *et al.*, 2010). The role of *Drosophila* Ced-6 has been demonstrated in phagocytosis and more recently in autophagy. The study described in this dissertation identifies a role of *Drosophila* Ced-6 during clathrin-mediated endocytosis.

1.4 Ovary development in *Drosophila*

I mentioned earlier that I used *Drosophila* ovaries to study clathrin-mediated endocytosis. Therefore, now I will discuss the events required for ovary development and thus essential for vitellogenesis. The egg chamber development of the *Drosophila* ovary is a powerful model to study numerous endocytic processes such as vesicular transport

activity, cell-polarity and endocytosis. The oocyte in the egg chamber of a *Drosophila* ovary is the largest cell in the organism. This single largest cell is not essential for survival of the adult fly, and therefore it can be easily manipulated.

Ovarian development and yolk protein accumulation is essential for embryogenesis and therefore for development into adult fly. Yolk protein accumulation in the egg chamber not only requires efficient endocytosis but also the polarization of oocyte egg assisted by the microtubules and Oskar-like polarity proteins. The aim of this part of the Introduction is to briefly discuss the main events of *Drosophila* oogenesis and then describe the ligands and receptors studied in this dissertation.

A female adult *Drosophila* possesses a pair of ovaries and each ovary consists of about 16 ovarioles. Each ovariole contains six or seven egg chambers at different developmental stages. Using morphological criteria, development of the egg chamber is divided into fourteen stages, stages 1-7 are previtellogenic, stages 8-11 are vitellogenic and stages 12-14 are the maturation stage (Cummings and King, 1970; King *et al.*, 1956) (Figure 1.5). In the *Drosophila* ovary, the stage 1 egg chamber develops into a mature egg in 3 days, while it takes seven days for a sixteen-cell cyst to develop into a stage 1 egg chamber (King, 1970).

Most *Drosophila* species reproduce during summer months and become reproductively inactive during the winter; this phenomenon is called reproductive or ovarian diapause. During the diapause, ovary development is halted at stage 7. Therefore, these ovaries contain egg chambers at previtellogenic stages 1-7. During diapause, ovaries do not accumulate yolk protein (Saunders et al., 1989b).

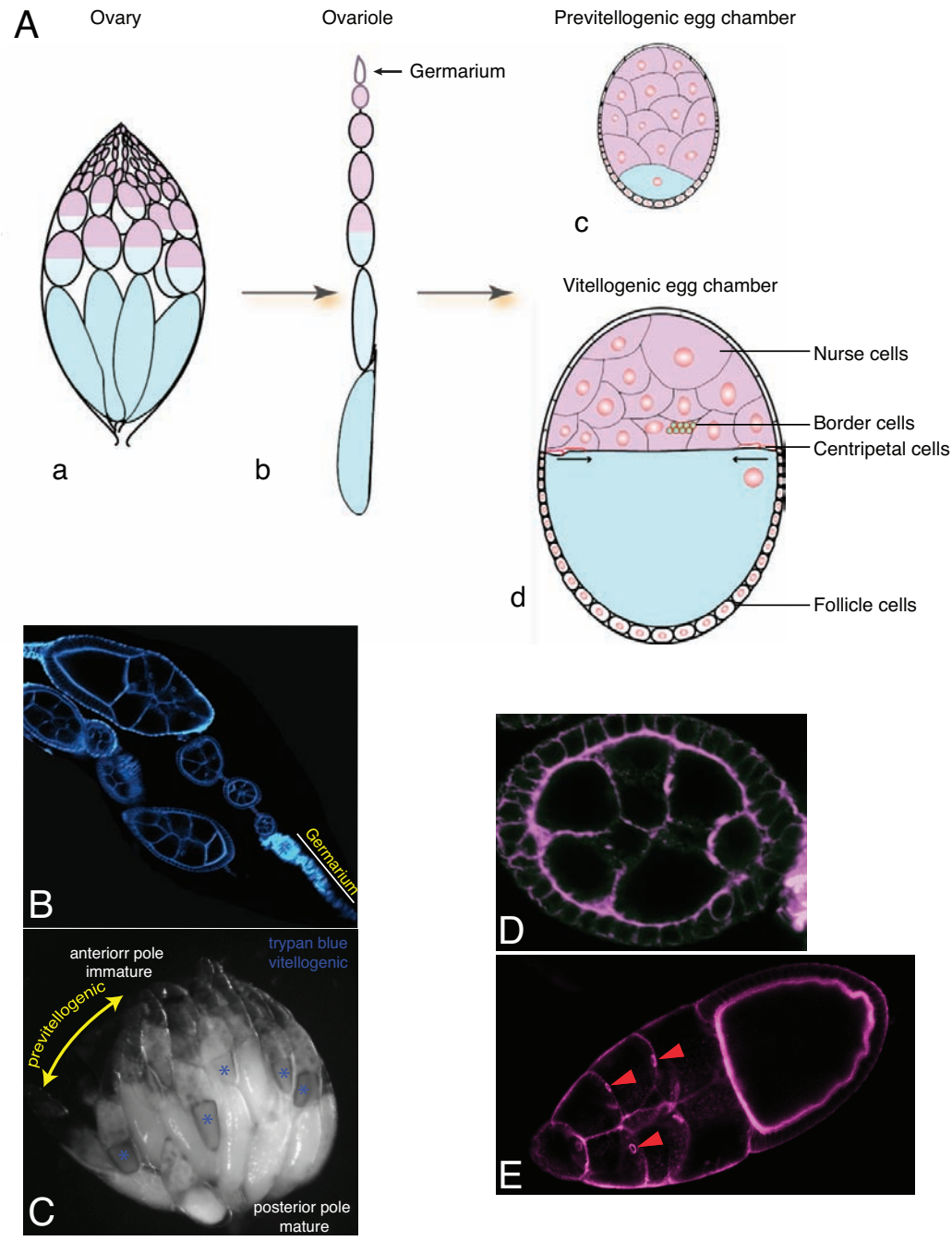


Figure 1.5: *Drosophila* ovary architecture.

(A) Schematic representation of a *Drosophila* ovary (a), an ovariole (b) and previtellogenic (c) and vitellogenic (d) egg chambers. (B, D and E) Single confocal sections of an ovariole stained with Phalloidin (D) stage 4 and (E) stage 10 egg chamber, red arrow showing the ring canal. (C) *Drosophila* ovary was bathed in trypan blue, showing the uptake of trypan blue by vitellogenic oocyte marked by blue asterisks. Original idea in (d) from (Ogienko et al., 2007).

1.4.1 Development of the egg chamber in the germarium

At the immature tip of each ovariole is a region called as a germarium. The germarium is the structure in which the production of the egg chamber is initiated. The germarium can be divided into four regions based on the morphology of the cyst (Figure 1.6). At the tip of each germarium, germline stem cells divide asymmetrically and produce a new stem cell and a cystoblast. The stem cells remain at the tip of the germarium, and the cystoblasts divide into sixteen cystocytes. At the end of the division process, one of the cystocytes becomes an oocyte and the remaining fifteen cells become nurse cells. The orientation of the first division of the cystoblast may determine the fate the cystocytes in becoming nurse cells or oocytes (Lin and Spradling, 1995). Another explanation for the fate decision is that during mitosis of cystoblasts, each of 16 cells is interconnected via a cytoplasmic bridge designated a ring canal. Two of the cystocytes contain four-ring canals and two cystocytes contain three-ring canals, while the rest contain two or one-ring canals. Either of the cystocytes containing four ring canals might be selected to become the oocyte (Figure 1.6). The ring canals allow the transfer of mRNA, proteins and organelle from nurse cells to the oocyte (Figure 1.5 E). When the 16-cell germline cyst is formed, somatic follicle cells migrate in and surround it. During oogenesis, the oocyte is arrested in prophase I of meiosis and becomes transcriptionally inactive, and the fifteen nurse cells are polyploid in nature. Thus the nurse cells, connected by the ring canals to oocyte, act as feeder cells to the oocyte.

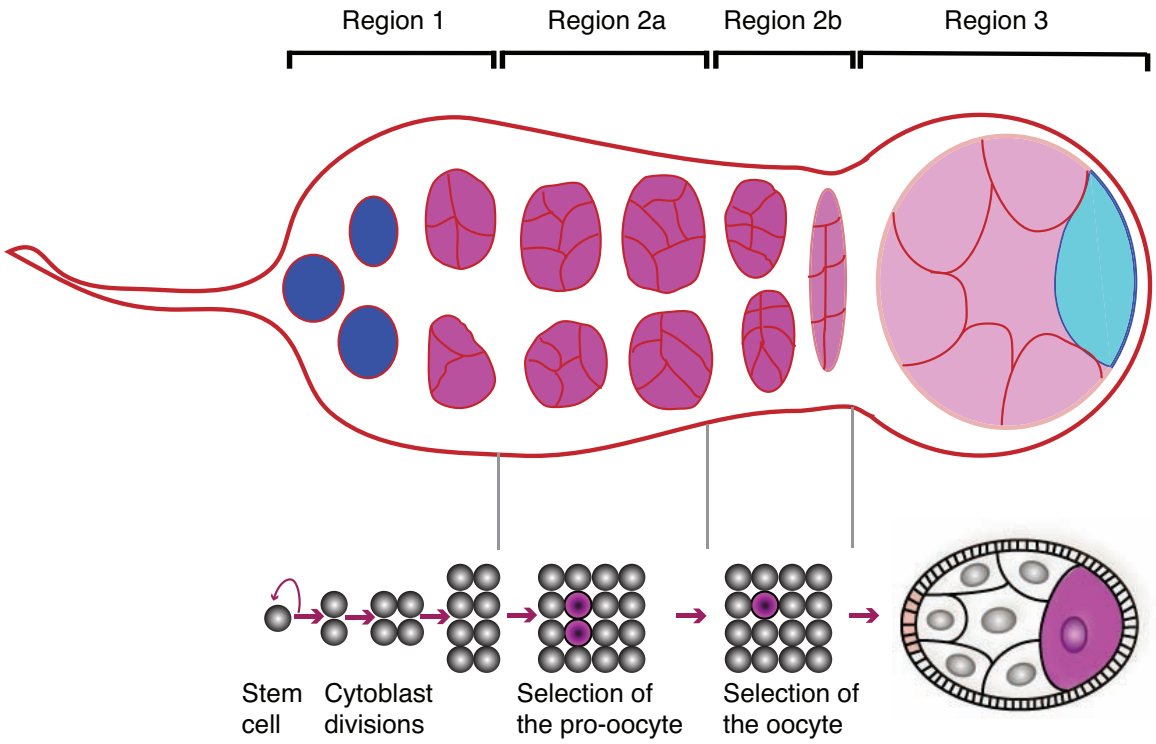


Figure 1.6: Germarium architecture and the emergence of the egg chamber.

Schematic representation of the *Drosophila* germarium organization (top) and asymmetric division of germline stem cells giving rise to single daughter cell with stem cell characteristics, and a cystoblast that will divide four times with incomplete cytokinesis to produce 15 nurse cells and one oocyte. Original idea from (Huynh and St Johnston, 2004).

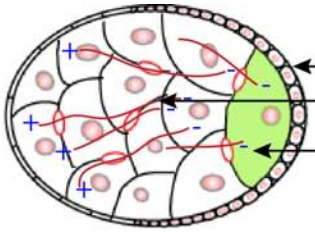
1.4.2 Development of the egg chamber outside the germarium

The egg chamber leaves the germarium at stage 1 of development. These egg chambers are separated from each other by the interfollicular somatic epithelium and a contractile meshwork called the peritoneal sheet, which covers the ovarioles (Middleton *et al.*, 2006).

In the egg chamber, the oocyte and nurse cells grow in size, and follicle cells divide by mitosis. In the germarium, somatic follicle cells are differentiated into three

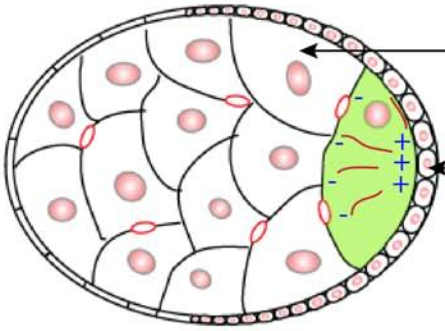
different cell types: polar, interfollicular and epithelial (Margolis *et al.*, 1995; Tworoger *et al.*, 1999). Epithelial follicle cells divide mitotically up to stage 6 of egg chamber development, and at stage 7 there are 650 follicle cells (Margolis *et al.*, 1995). Until stage 6 of egg chamber development, epithelial follicle cells are roughly uniform in shape and size (Figure 1.5 D). At stages 7 and 8 most of the follicle cells migrate toward the posterior side of the growing oocyte and become cylindrical in shape (Horne-Badovinac and Bilder, 2005; Spradling, 1993a) as shown in Figure 1.5 A [d] and E. At stage 9 of egg chamber development, a separate group of cells, the polar epithelial cells, migrate through the germline nurse cells towards the oocyte; these follicle cells are known as border cells (Montell *et al.*, 1992). The migration of polar cell is also used to define the stage of egg chamber development (Figure 1.5 A [d]). At stage 10, the follicle cells, which now cover mostly the oocyte, are cylindrical; the follicle cells associated with nurse cells are flattened and expanded and form a thin layer of epithelium (Figure 1.5 A [d]). During stage 10, cylindrical follicle cells (centripetal cells), which are near the nurse cells, migrate and become flattened then fully cover the anterior part of the oocyte between the nurse cells (Figure 1.5 A [d]). Also at the end of oogenesis, the follicle cells secrete substances to produce the egg shell. Follicle cells surrounding the oocyte also play an important role in determining the polarity, the anterior to posterior and dorsal to ventral axes of the embryo (Horne-Badovinac and Bilder, 2005).

Stage 6



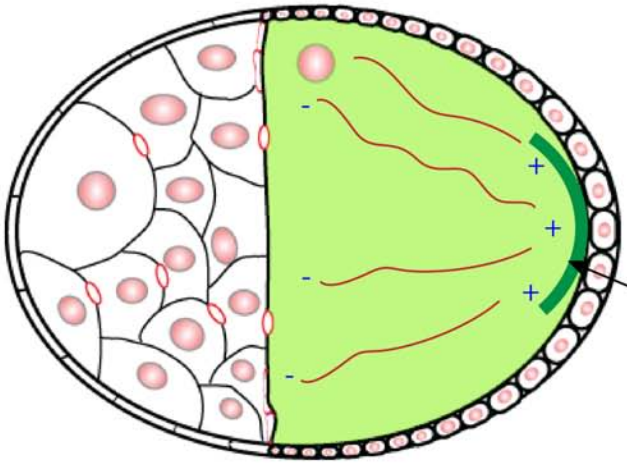
Oocyte
Microtubule
Ring canal

Stage 8



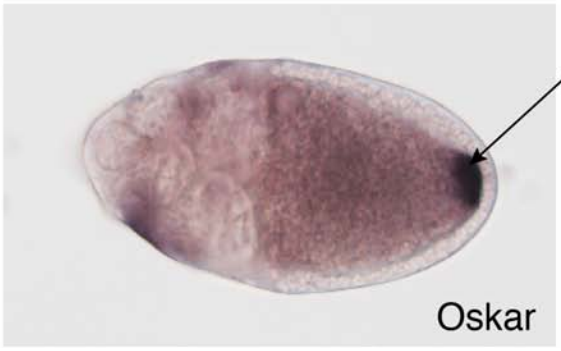
Nurse cells
Follicle cells

Stage 10



Pole plasm

Anterior ⟷ Posterior



Pole plasm

Figure 1.7: Schematic illustration of microtubule-assisted nurse cell cytoplasm transport.

Microtuble-assisted cytoplasm transport illustrated in a stage 6-10 oocyte through ring canals. The plus end of microtubule is in the nurse cells until stage 6, and microtubule polarity is reversed during stage 8 and 10. Bottom panel showing *oskar* mRNA in a stage 10 oocyte in the pole plasm. Original idea from (Tanaka and Nakamura, 2011).

1.4.3 Transport of nurse cell cytoplasm into the oocyte

At the early stage of egg chamber development, transport of different molecules from the nurse cells to the oocyte is very slow and selective for certain mRNAs, proteins and organelles. The microtubule cytoskeleton assists in the selective transport and, until stage 6, the plus end of the microtubule is positioned in the nurse cell (Figure 1.7). Studies done to detect the microtubule organizing center (MTOC) in the early stage of oocyte development have shown the role of nurse cell centrosome migration to oocyte in microtubule organization (Gonzalez C., 1998). The intracellular signaling between follicle cells and oocyte reorganizes the polarity of microtubule at stages 6-7 (Figure 1.7). After this stage, the plus end of the microtubules is towards the posterior region of oocyte. Oocyte polarization and positioning of the oocyte nucleus depends on motor proteins such as dynein and kinesin I. At stage 10, rapid transport starts and most of the nurse cell contents are transferred into the oocyte; this rapid transport is known as cytoplasmic dumping. After dumping, nurse cells are left with nuclei, some actin filaments and a minor amount of cytoplasm (Spradling, 1993a; Spradling, 1993b). Microtubule formation and reorganization is necessary for oocyte growth and development, and disruption of microtubule formation greatly affects oocyte development (Koch and Spitzer, 1983).

In several animals, the inheritance of polar granules (like the cytoplasmic granules localized at the posterior end of the *Drosophila* oocyte and embryo) during the egg chamber development is essential for later germ cell development. During stage 9, the oocyte starts the formation of the precursor to polar granules, which are in turn clearly present during stage 10 (Mahowald, 1963; Mahowald, 1971). Vasa, Oskar and Tudor proteins and mitochondrial 16S large rRNA (mthRNA) are thought to be components of the polar granules in pole plasm (Bardsley *et al.*, 1993; Kobayashi and Okada, 1988).

1.4.4 Oskar

The Oskar protein has a critical function in pole plasm assembly (Figure 1.7). Interestingly recent studies have also shown a significant role of Oskar in endocytosis. The localization of *oskar* mRNA and its translation is sufficient to activate pole plasm assembly. The so-called short and long isoforms of Oskar show different subcellular localizations as determined by immunoelectron microscopy. The long isoform of Oskar is found in endocytic vesicular structures, and short Oskar is found on specialized ribonucleoprotein aggregates of pole plasm (Vanzo *et al.*, 2007). *Oskar*-null oocytes accumulate less yolk-protein than the wild-type oocytes. (Vanzo *et al*., 2007) At this stage of development the endocytic pathway is not only required for yolk protein accumulation in the oocyte, but also for polarization of the microtubule. In the absence of Rabenosyn-5 (expressed from the *rbsn-5* gene) or in *rab5* mutants, pole plasm components, including Oskar, diffuse throughout the cytoplasm rather than localizing in the posterior end as they do in normal oocytes (Tanaka *et al.*, 2011; Tanaka and Nakamura, 2008). These studies also indicate that Oskar-dependent actin remodeling is involved in the endocytic pathway.

Thus the endocytosis at the posterior pole to form polarity and the polarization to enhance the endocytic activity at the posterior pole are interdependent processes.

1.4.5 Cell death at the end of oogenesis

At the end of oogenesis, when nurse cells dump their cytoplasmic contents into the oocyte, the remnants of the nurse cells are engulfed by the somatic follicle cells (Cummings and King, 1970; Nezis *et al.*, 2000). Nurse cell death and death of salivary gland cell in the larva are similar in several ways (Baehrecke, 2003). Salivary cells also die in a cluster like nurse cells, and salivary gland cell death is also initiated by ecdysone (McCall, 2004). Nurse cells contain an autophagic vacuole near the Golgi (Witkus *et al.*, 1980). Cooperative action of apoptosis and autophagy might be responsible for cell death in nurse cells. Anterior follicle cells at stage 14 of oogenesis contain vacuoles filled with organelles and cytoplasmic material (Nezis *et al.*, 2006a). The follicle cells also secrete chorion, and after chorion synthesis is complete, the fully mature egg chamber exits from the ovariole. At this time, the dying follicle cell detach from the egg outer surface and are phagocytosed by the epithelial layer of the oviduct (Nezis *et al.*, 2006a).

As mentioned previously, the egg chamber is at stage 1 when it leaves the germarium, and at stage 14 it is ready for fertilization. After fertilization, nuclear division occurs in the absence of cytokinesis. Immediately after fertilization, the Oskar-containing polar granules are organized in a cluster and associate with mitochondria. During the mitotic cycle of embryogenesis, approximately eight to ten nuclei reach the posterior pole and become surrounded by cell membrane. These cells further divide non-synchronously to produce 40 pole cells (Sonnenblick, 1948).

Germ cells originate from pole cells and contain a cytoplasm that is enriched with dense polar granules, RNAs and proteins different in composition than those of somatic cells. In females, these germ cells divide by mitosis; this process occurs at the larval stage, and during the third instar of larval stage these germ cells differentiate into ovaries (King, 1970).

Oogenesis does not advance into embryogenesis until a certain amount of yolk protein has been accumulated (Bownes et al., 1991). During the oogenesis, large amount of yolk-protein must be accumulated in the oocyte, which can provide nutritional support during the embryogenesis (Bownes, 1986).

1.5 Yolk protein

Yolk protein accumulation in the oocyte of a *Drosophila* is a heterosynthetic process, because the majority of yolk protein is synthesized by the fat bodies and transported to oocytes (Postlethwait and Giorgi, 1985). In insects, the fat body is a loose tissue localized underneath the cuticle surrounding the gut and reproductive organs. Fat bodies are organized in thin lobes, which are bathed by hemolymph (Arrese and Soulages, 2010). The precursor of yolk protein is called vitellogenin, and after the uptake and storage in the oocyte; vitellogenins are called vitellins (Izumi *et al.*, 1994). The fat-body secreted yolk proteins are internalized by the oocyte with the Yolkless receptor (DiMario and Mahowald, 1987; Schonbaum *et al.*, 1995).

In *Drosophila*, a region on the X chromosome encodes three yolk proteins (YP1, YP2 and YP3) (Butterworth *et al.*, 1999). In addition to the fat body YP1, 2 and 3 are transcribed in somatic follicle cells as well. As previously mentioned, follicle cells of the ovary stop mitotically dividing at stage 6 and continue to synthesize mRNA until stage

14. The yolk protein transcripts can be detected from stage 8 until stage 10B (Brennan *et al.*, 1982), covering the period of active yolk capture by the oocyte. Consequently, the expression of yolk protein transcripts is regulated by different upstream regions in follicle cells and fat bodies (Bownes, 1986).

Unlike the large vitellogenin in most insects, the vitellogenin from higher diptera like *Drosophila* are small (-40 kDa) (Tufail and Takeda, 2009). On SDS-polyacrylamide gels, yolk proteins appear as a triplet with molecular weights of 44 kDa, 45 kDa and 46 kDa. Each of the yolk proteins has a slightly different isoelectric point. Limited proteolysis results in different digestion patterns for the three yolk proteins (Mahowald *et al.*, 1981). Although the primary structures of yolk proteins do not show high conservation, the secondary structure does and this leads to common activity (Hung and Wensink, 1983).

The Yolkless receptor binds to yolk protein in *Drosophila* oocytes; this receptor-ligand complex is sequestered in coated-pits for endocytosis. After internalization, the yolk proteins are stored in yolk spheres/yolk granules (Figure 1.8). These yolk spheres are endosome-like structures that fuse with Golgi-derived vesicles (Giorgi *et al.*, 1993) (Figure 1.8). The internalized yolk protein is stored in yolk spheres for later use in development or targeted for degradation in lysosomes; the fate is dependent on the hormonal status of the oocyte (Giorgi *et al.*, 1993) (Figure 1.8).

It is proposed that two molecules of vitellogenin can bind to one molecule of vitellogenin receptor in piscine species (Li *et al.*, 2003). It was determined that one LDL particle can bind to one LDLR (Jeon and Blacklow, 2005; Van Driel et al., 1989). The size of the coated pit varies depending on its constituents: Plants have very small coated-pits (Dhonukshe *et al.*, 2007), and the coated pits in chicken oocytes are about ~150 nm

in diameter (McMahon and Boucrot, 2011; Perry and Gilbert, 1979). The precise number of yolk proteins bound to a single Yolkless receptor is not known, but certainly the coated pits in *Drosophila* oocytes are quite large (Figure 1.8).

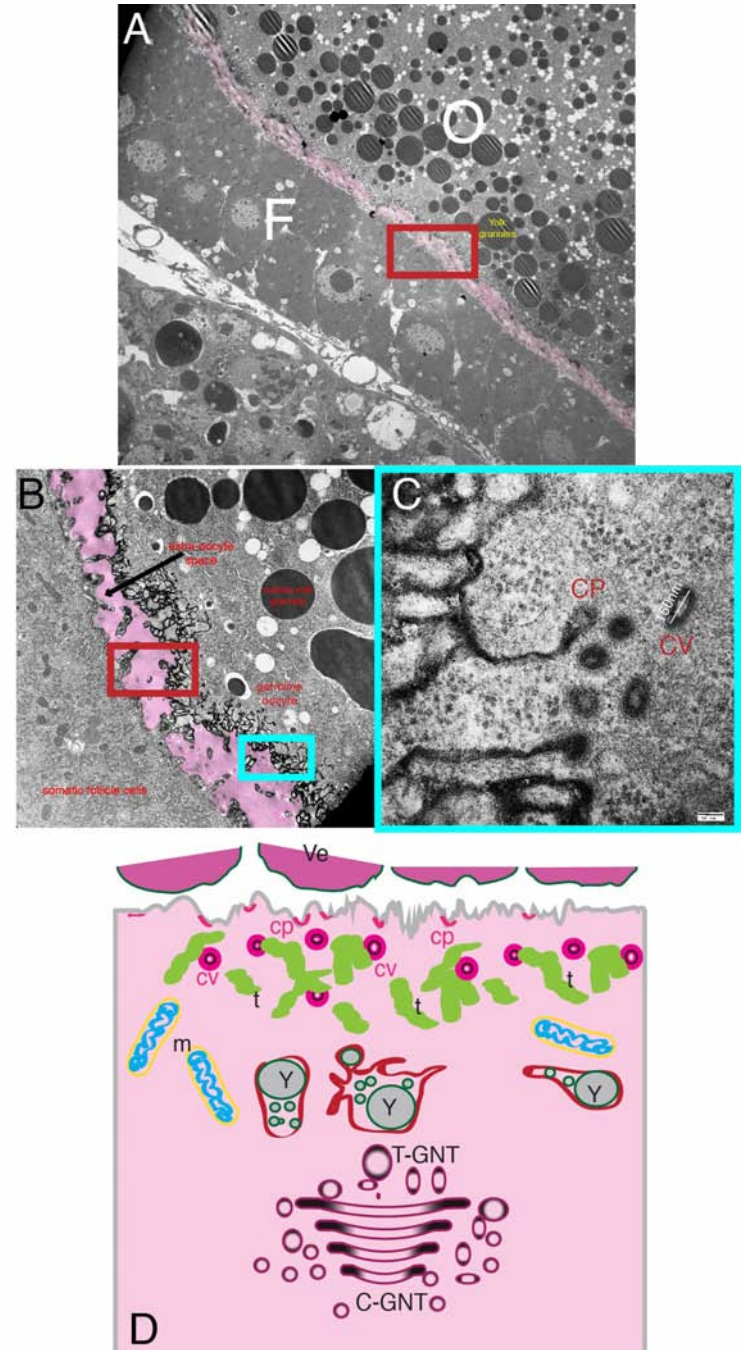


Figure 1.8: Electron microscope images of the stage 10 oocyte.

(A) High-resolution image of a stage 10 oocyte showing the follicle cells (F) and deposition of yolk granules in oocyte (O). (B) The extra oocyte space is indicated in pink and separats the somatic follicle cells and the germline oocyte. (C) The coated pits (CP) and coated-vesicles (CV) (about 150 nm in size). (D) Schematic illustration of cortical endocytic zone of a vitellogenic oocyte of *Drosophila*. cp, coated pits; cv, coated vesicles; C-GNT, cis Golgi network; m, mitochondria; t, tubules; T-GNT, trans-Golgi network; Ve, vitelline envelope and Y, yolk spheres. Original idea was from (Giorgi et al., 1993).

1.6 Yolkless

As mentioned earlier, in insects, including *Drosophila*, most of the yolk protein precursor is synthesized in fat bodies and then secreted into the hemolymph, where it is captured by membrane-bound receptors in the oocyte. These receptors accumulate yolk protein with the help of receptor-mediated endocytosis. Several yolk protein precursors accumulate in the oocyte, but vitellogenin is the most important and abundant precursor. Generally the receptor for vitellogenin uptake is known as the vitellogenin receptor (VgR). These vitellogenin receptors are members of the LDLR superfamily. The *Drosophila* vitellogenin receptor is known as the Yolkless receptor. The *Drosophila* oocytes from homozygous null allele of Yolkless receptor have very little yolk protein in the oocyte (DiMario and Mahowald, 1987). There is a high degree of structural and functional similarity between different vitellogenin receptors; however, in insects the VgR gene transcript is ~7 kb and encodes for ~200 kDa protein. Duplication of part of the luminal ligand binding domain roughly increases the size twice to those for vertebrate VgRs (~95kda) (Tufail and Takeda, 2007). A growing oocyte also internalizes lipophorin, arylphorin and microvitellogenin (Kawooya *et al.*, 1988; Kulakosky and Telfer, 1990; Machado *et al.*, 1996).

These insect VgRs/LpRs mirror the structural organization of LDLR. LDLR superfamily members have five structural motifs: (1) a ligand-binding domain (LBD), (2) an EGF precursor domain (EGFD), (3) an O-linked sugar domain (OLSD), (4) a transmembrane domain (TMD) and (5) a cytoplasmic domain (Figure 1.9). A different arrangement of the structural units can result in altered ligand recognition (Hobbs *et al.*, 1990). One of the more common properties of LDLR family members is binding of receptor-associated protein (RAP), which is present in the endoplasmic reticulum and acts

as a chaperone for proper folding of receptors and prevents inappropriate ligand binding within the biosynthetic pathway.

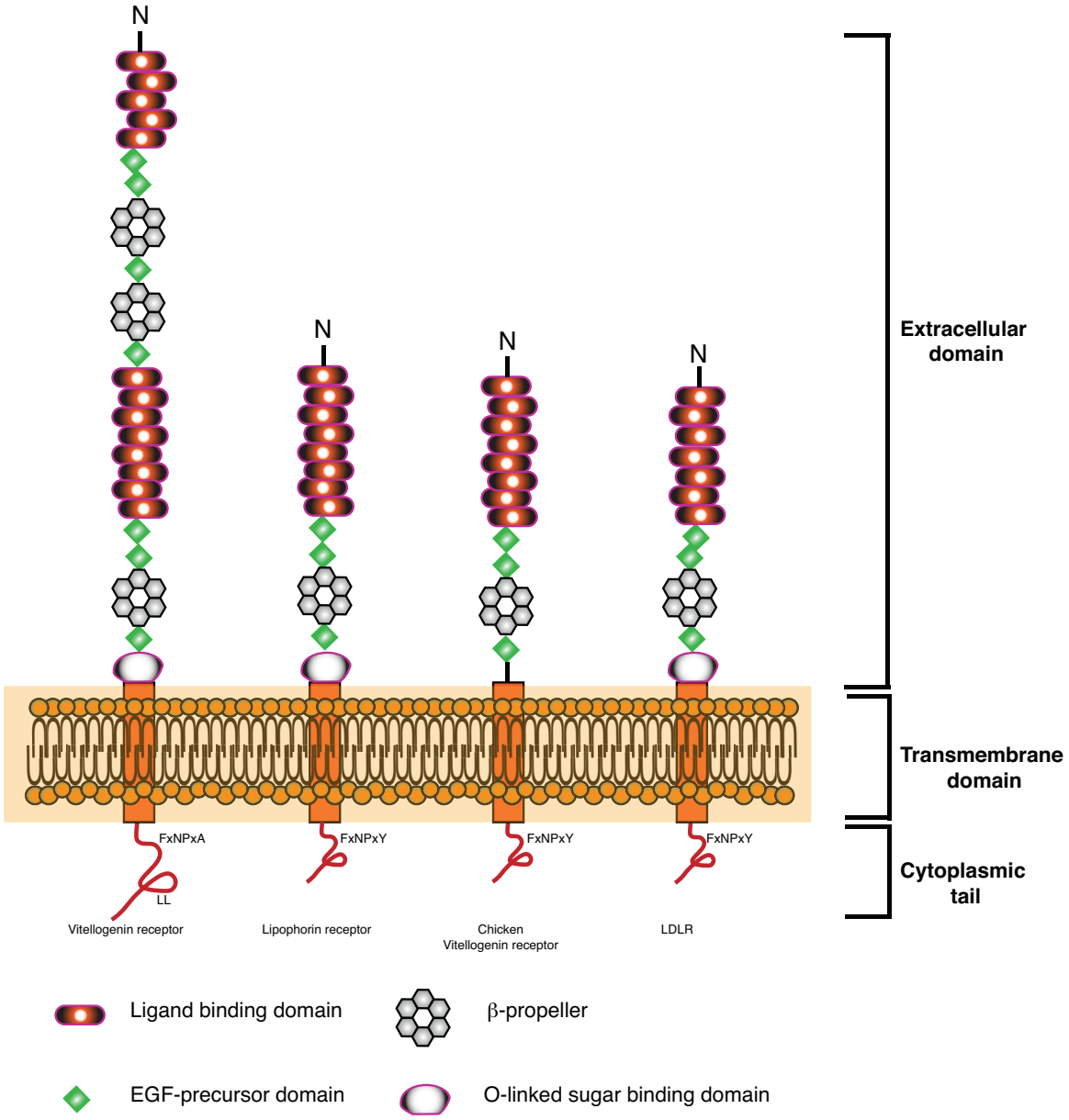


Figure 1.9: Modular structure of LDL receptor family members.

LDL receptor is compared to vitellogenin receptor, lipophorin receptor and chicken vitellogenin receptor; extracellular domain (ligand binding domain), EGF-precursor domain, β-propeller and O-linked sugar binding domains, transmembrane domain and cytoplasmic tail with essential sorting motifs are indicated. Original idea was from (Tufail and Takeda, 2009).

In vertebrates, normally the LDLR binds to its ligand at the cell surface at neutral pH. The receptor-ligand complex is internalized by clathrin-coated pits. In the endosome, this receptor-ligand complex is then dissociated and the receptor is returned to the surface with the help of the recycling endosome (Brown and Goldstein, 1986). The ligand-binding region of LDLR is made up of six cysteine-rich repeats of ~40 amino acids. There are two separate ligand-binding domains within the insect vitellogenin receptor (Figure 1.9): The first region contains five repeats and the second region consists of eight repeats (Tufail and Takeda, 2009). In mammalian LDLRs, the ligand-binding domain consists of only seven cysteine-rich repeats (Figure 1.9). Therefore, the lipophorin receptor of *Drosophila* with one ligand-binding domain shows a high similarity to mammalian LDLR (Tufail and Takeda, 2009).

The ligand binding domain is followed by the EGF precursor domain in the LDLR superfamily. EGF precursor domain contains EGF-like repeats and a β-propeller domain-containing YTWD motif repeats. The insect vitellogenin receptor contains seven EGF-like repeats. Mutation of any of these repeats affects the pH-dependent the dissociation of the ligand from the receptor within the endosome (Davis *et al.*, 1987). However, the insect lipophorin receptor was found to remain bound to ligand in the endosome. These findings suggested ligand recycling by the lipophorin receptor, similar to recycling by the

transferrin receptor in vertebrates (Dantuma *et al.*, 1997; Roosendaal *et al.*, 2008; Van Hoof *et al.*, 2003).

Next to the EGF precursor domain in LDL receptor superfamily members is the O-linked sugar domain . The O-linked sugar domain is enriched in serine and threonine residues, and many of these residues are O-glycosylated (Willnow, 1999). The insect vitellogenin receptor O-linked sugar domain is ~30 residues long. However, the O-linked sugar domain is absent from the *Drosophila* vitellogenin receptor (Schonbaum *et al.*, 1995). On the carboxy-terminal end of the O-linked sugar doamin, the transmembrane domain begins. The transmembrane domain forms an α-helix and is involved in the anchoring of LDLR members to the plasma membrane (Herz and Bock, 2002; Willnow, 1999). At the very end of the carboxy-terminus of the LDLR family lies the cytoplasmic domain. The cytoplasmic domain of LDLR family contains the NPxY sequence, which is an internalization sequence recognized by the clathrin-coated pit (Chen *et al.*, 1990; Morris and Cooper, 2001). LDLR and VLDLR each contain one NPxY signal, whereas LRP1 and Megalin each contain two copies of the NPxY signal. LRP1 also contains an YxxL motif (Li *et al.*, 2000). LRP3, LRP5 and LRP6 harbor an YxxL and/or a dileucine motif for endocytosis. The vitellogenin receptors from mosquito and *Drosophila* harbor NPxL and NPxA motifs, respectively.

Vitellogenin receptors are only expressed in the ovary (Schneider, 1996). The expression pattern and subcellular distribution of the mRNA and protein provide important clues to vitellogenin receptor regulation (Schonbaum *et al.,* 2000). In *Drosophila*, the *vitellogenin receptor* transcript is localized to nurse cells and oocytes within the ovary (Schonbaum *et al.*, 2000) (Appendix Figure A.3).

1.7 Summary of Introduction and research goals

Endocytosis is essential for both cellular housekeeping and for specialized functions. The accumulation of yolk protein in the oocyte is an absolute necessity for embryogenesis and development into the adult fly. The oocyte is a germ cell in the egg chamber and its development is dependent on several factors. One is transport of nurse cell contents into oocyte, since the oocyte is transcriptionally inactive. Follicle cells are somatic cells and are important for development of the oocyte and, at later stages of development for the engulfment of degenerated nurse cells. The follicle cells and fat-body cells synthesize the yolk protein precursor and yolk protein is secreted in the hemolymph. An understanding of the mechanism of yolk protein secretion in to the hemolymph and subsequent internalization by the Yolkless receptor leading to storage as a yolk sphere was a necessary first step in the understanding of yolk protein endocytosis in the *Drosophila* oocyte.

Clathrin-mediated endocytosis oversees a yolk protein and Yolkless complex internalization into the oocyte. The highly conserved proteins required for this highly orchestrated event have been studied in different tissues of several organisms. Research done by several groups has established the importance of sorting signals and essential motifs carried by clathrin-associated proteins. The main focus of this dissertation research was to understand the molecular mechanism of Yolkless endocytosis during the vitellogenesis and relate this new information to the body of established work in the field of endocytosis. Knowledge of the mechanism of egg chamber development during oogenesis is an absolute requirement for understanding of Yolkless endocytosis and the interdependence of polarity and endocytosis.

The goal of this dissertation was therefore to identify the required sorting signals present in the cytosolic tail of Yolkless. I found that the Yolkless cytosolic tail contains two sorting signals that mediate internalization by clathrin-mediated endocytosis. The Yolkless cytoplasmic tail has an FxNPxA sequence and a non-canonical dileucine sequence that both serve as separate internalization signals. The experiments carried out to find the associated proteins required to recognize these signals revealed the involvement of a phagocytic protein in clathrin-mediated endocytosis. I found that Ced-6 fulfills all the criteria to be a designated CLASP. Ced-6 interacts with the Yolkless cytosolic tail and assists in Yolkless internalization. *Ced-6* null *Drosophila* show some defects in internalization of yolk protein. In mammals, Dab2 and ARH play redundant roles in LDLR endocytosis, therefore other adaptor proteins may have similar redundant functions in Yolkless trafficking in *Drosophila*. I have also studied AP-2 function in Yolkless internalization. AP-2 binds to the non-canonical dileucine sequence of Yolkless. Therefore both Ced-6 and AP-2 have important functions in Yolkless endocytosis. Ced-6 was previously shown to bind to the phagocytic receptor and is involved in phagocytosis of neurons (Awasaki et al., 2006). The mammalian homolog of Ced-6 is Gulp. I have found that Gulp is also present in clathrin-coated pits. This finding enabled us to propose a significant and possibly necessary role for clathrin-mediated endocytosis in phagocytosis.

2.0 YOLK ENDOCYTOSIS BY CLATHRIN: A TASK SHARED BY CED-6 AND AP-2 IN THE *DROSOPHILA* EGG CHAMBER

2.1 Introduction

In all the oviparous animals, including insects, yolk protein accumulation is necessary for proper embryonic development. Early stage embryonic development occurs without any external or maternal assistance; therefore meeting the energy requirement for progeny development depends on yolk protein storage. In insects the precursor of yolk protein, vitellogenin, is synthesized outside of the ovary by the fat body. After post-translational modification, yolk protein is secreted into the hemolymph. It is then captured from circulating hemolymph by receptors and endocytosed into the egg chamber (Raikhel and Dhadialla, 1992; Tufail and Takeda, 2008). The first description of endocytosis by clathrin-coated vesicles came from the study of vitellogenin uptake in mosquito oocytes (Roth and Porter, 1964). Vitellogenesis in insects is regulated by hormones, food and the stage of egg-chamber development (Bownes, 1986).

In mosquitoes, a blood meal is required for the synthesis of vitellogenin and the lipoprotein-like transporter lipophorin by the fat body (Cho and Raikhel, 2001). Vitellogenin receptor levels are also upregulated after a blood meal, resulting in an increased degree of receptor-mediated capture of vitellogenin and subsequent internalization by clathrin-mediated endocytosis. Vitellogenin receptors are members of the LDLR super-family, which typically harbor the [FY]xNPx[YF] sorting signal that directs clustering into the clathrin coat. An N-terminal phosphotyrosine-binding (PTB) domain containing protein can recognize these signals and concentrate the receptors into

the assembling clathrin coat (Mishra et al., 2002a; Mishra et al., 2002b; Traub, 2009). These PTB-domain-containing proteins can physically interact with receptors, phosphatidylinositol 4,5-bisphosphate (Ptdins(4,5)P_2), heterotetrameric AP-2 clathrin adaptors and clathrin. They are also members of the family of clathrin-associated sorting-adaptor proteins (CLASPs) (Traub, 2009). Mosquitoes express trephin, a PTB domain containing protein that is a mammalian homolog of ARH or LDLRAP1, and is transcriptionally regulated by a blood meal (Mishra *et al.*, 2008).

Mosquito oocyte development depends highly on a blood meal, whereas *Drosophila* ovary development occurs asynchronously and depends on environmental cues such as temperature, duration of light and food (Bownes, 1986). The entire ovary is bathed in hemolymph continuously, yet the uptake of yolk protein occurs only in vitellogenic stages (Mahowald, 1972a). Egg chambers at these yolk storing stages show cortical clathrin-coated structures, which are nearly absent in the pre-vitellogenic stages (Tedesco, 1981). Three lines of evidence suggest the involvement of clathrin-dependent endocytosis in *Drosophila* yolk protein storage. First, yolk uptake is disrupted in the *shibire* mutant, a temperature-sensitive form of the large endocytic GTPase dynamins. Temperature shock above ambient temperature (29˚C) for 1-5 minutes causes the disappearance of vesicles and membrane tubules from the cortical region of the oocyte (Kessell *et al.*, 1989). These data confirm that clathrin-mediated endocytosis is also affected in the *shibire* mutant. Second, it is well established that the human transferrin receptor is internalized by clathrin-mediated endocytosis. The cytosolic tail of the receptor is recognized by the heterotetrameric adaptor protein AP-2 and concentrated into clathrin-coated pits. Human transferrin receptor has been ectopically expressed in fly oocytes and shown to be internalized (Bretscher, 1996). In the *shibire* background, at

restrictive temperature, transferrin receptor is mainly found at the plasma membrane of the oocyte and not in the vesicular structures (Bretscher, 1996). This finding further establishes the role of clathrin-mediated endocytosis in oocytes. Third, a female-sterile-mutation screen in *Drosophila* revealed a vitellogenic receptor ortholog designated as *yolkless* (DiMario and Mahowald, 1987). Homozygous *yolkless* mutant flies lay flaccid eggs with little or no yolk protein. The cortex of these *yolkless* mutant flies showed ~10-fold decrease in clathrin-coated structures relative to the wild-type flies (DiMario and Mahowald, 1987). These studies clearly suggest a role of clathrin-mediated endocytosis in yolk storage.

It was not known precisely how Yolkless was internalized by clathrin-mediated endocytosis. In this study I identified interaction partners for Yolkless endocytosis. The Yolkless cytosolic tail contains two atypical sorting signals that are conserved across *Drosophila* species. Moreover, *Drosophila* does not express an ARH ortholog like the mosquito, indicating that *Drosophila* might be using an alternative CLASP that can interact with the atypical sorting signals of Yolkless and concentrate it into the clathrin-coated pits. In the present study, I have found that – along with AP-2 – Ced-6, a PTB domain containing protein, functions as a CLASP for Yolkless uptake in clathrin-coated structures.

2.2 Results

2.2.1 Endocytic stages of oogenesis

In *Drosophila melanogaster*, yolk storage is essential for oocyte maturation and it occurs between stages 8 and 11, where stages 9 and 10 are the main vitellogenic stages (Mahowald, 1972b) (Figure 2.1 A and B). This endocytic process lasts for ~16 hours

(King, 1970). During vitellogenesis (yolk storage), the oocyte and nurse cells increase in volume and the oocyte accumulates large amounts of yolk proteins and neutral lipids that are captured from the surrounding hemolymph (Parra-Peralbo and Culi, 2011; Spradling, 1993a). The Yolkless receptor mediates the endocytic uptake of yolk proteins (DiMario and Mahowald, 1987). In the present study, uptake of ligand by vitellogenic receptor was observed using monomeric Cherry red fluorescent protein tagged receptor-associated protein (mcRFP-RAP), an ER chaperone used as a pseudo-ligand for LDL receptor super-family members (Figure 2.1 B and D). In vitellogenic chambers, where yolk protein endocytosis occurs, Yolkless receptor expression was shown to become cortical (Figure 2.1 C). In fact, receptor localization to the cortex of the oocyte coincided with the uptake of mcRFP-RAP. During stage 8 of egg chamber development, receptors started to be localized at the oocyte membrane as visualized with immunostaining of Yolkless, which indicates uptake of mcRFP-RAP. During the highly endocytic stage of oocyte development (stage 10), Yolkless was mainly localized to the oocyte membrane and there was a high level of accumulation of mcRFP-RAP, which was stored along with yolk protein in more central, larger mature yolk granules and spheres (Figure 2.1 C). To observe clathrin expression in oocytes, the clathrin light chain was cloned along with GFP into the pUASP vector. Clathrin was observed mainly within internal structures during the pre-vitellogenic stages (Figure 2.1 E), but it also relocated to the cortical site (oocyte membrane) during vitellogenesis (Figure 2.1 F). These morphologic data correlate the location of Yolkless and clathrin during vitellogenesis.

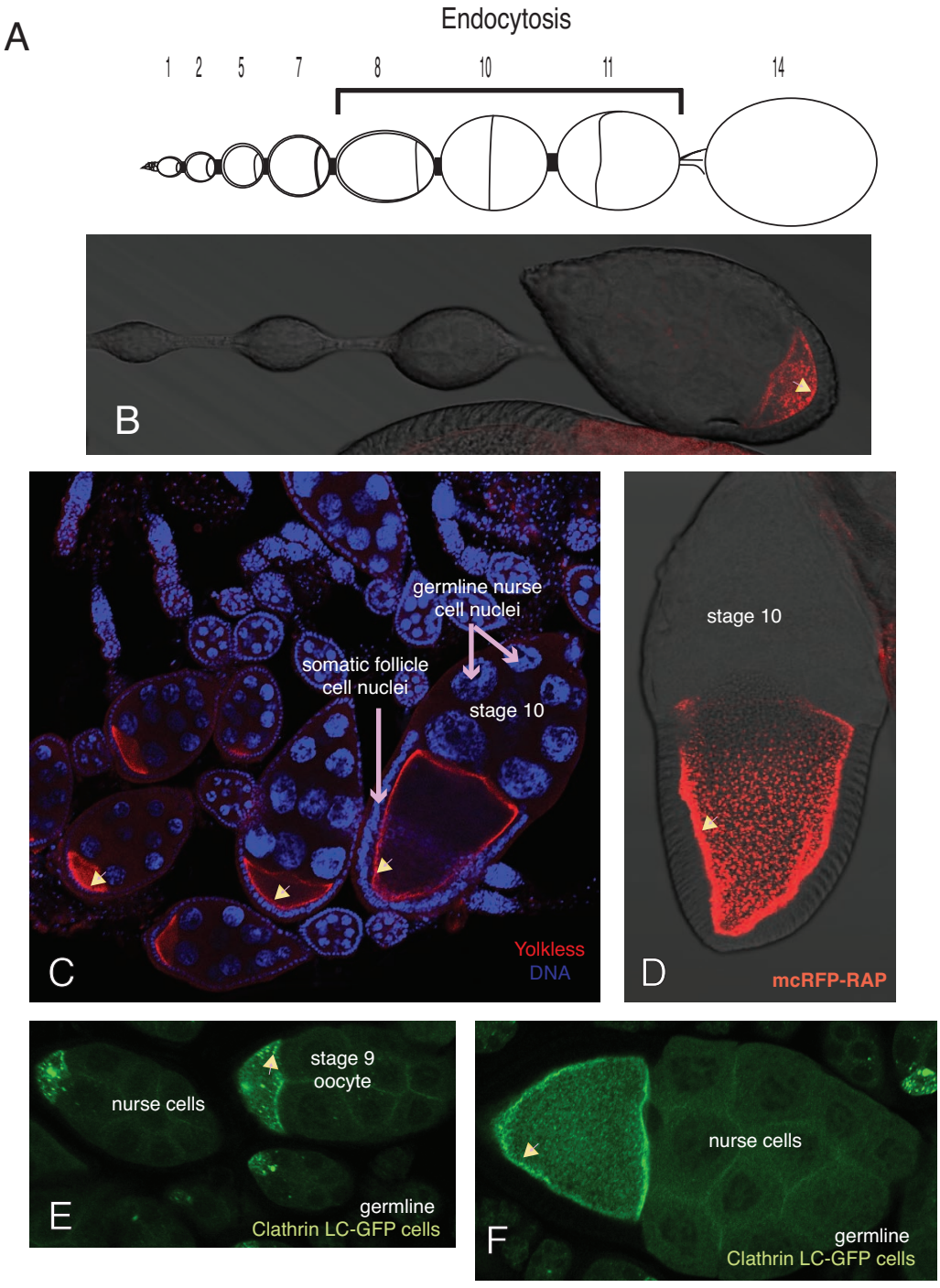


Figure 2.1: Receptor-mediated endocytosis in *Drosophila* oocytes.

(A) Schematic illustration of different stages of *Drosophila* oocyte development. Original idea for this figure was from King, 1970. (B and D) Receptor-mediated internalization of GST-mCherry-RAP in oocyte. (C) Expression of Yolkless in oocyte (in red) and DAPI (in blue). Over-expression of CLC-GFP (yellow arrow heads) in previtellogeic (E) and vitellogenic (F) *Drosophila* oocytes.

2.2.2 Endocytosis in pre-vitellogenic (diapausal) and vitellogenic ovaries

Unfavorable conditions related to temperature, light and food can cause reproductive diapause in *Drosophila* (Gilbert et al., 1998; Saunders et al., 1989a). During reproductive diapause, ovariole development is halted at a pre-vitellogenic stage to conserve energy (Gilbert *et al.*, 1998). To create a diapausal condition, *Drosophila* were kept at 11˚C and given 14 hours of darkness and 10 hours of light. Harvested ovaries under diapausal and vitellogenic conditions were incubated with mcRFP-RAP. Previtellogenic fly ovaries showed no uptake of mcRFP-RAP, while vitellogenic ovaries not only showed oocyte development morphologically, but also efficiently internalized mc-RFP-RAP (Figure 2.2 A). To observe the levels of various endocytic proteins under these conditions, equal numbers of ovaries from diapausal and vitellogenic females were collected to make ovary lysates, and the components were separated by SDS-PAGE. Coomassie-stained gels showed different levels of protein expression in diapausal *vs*. vitelloginc conditions. More specifically, ~45 KDa yolk proteins accumulated massively in the vitellogenic ovary (Figure 2.2 B).

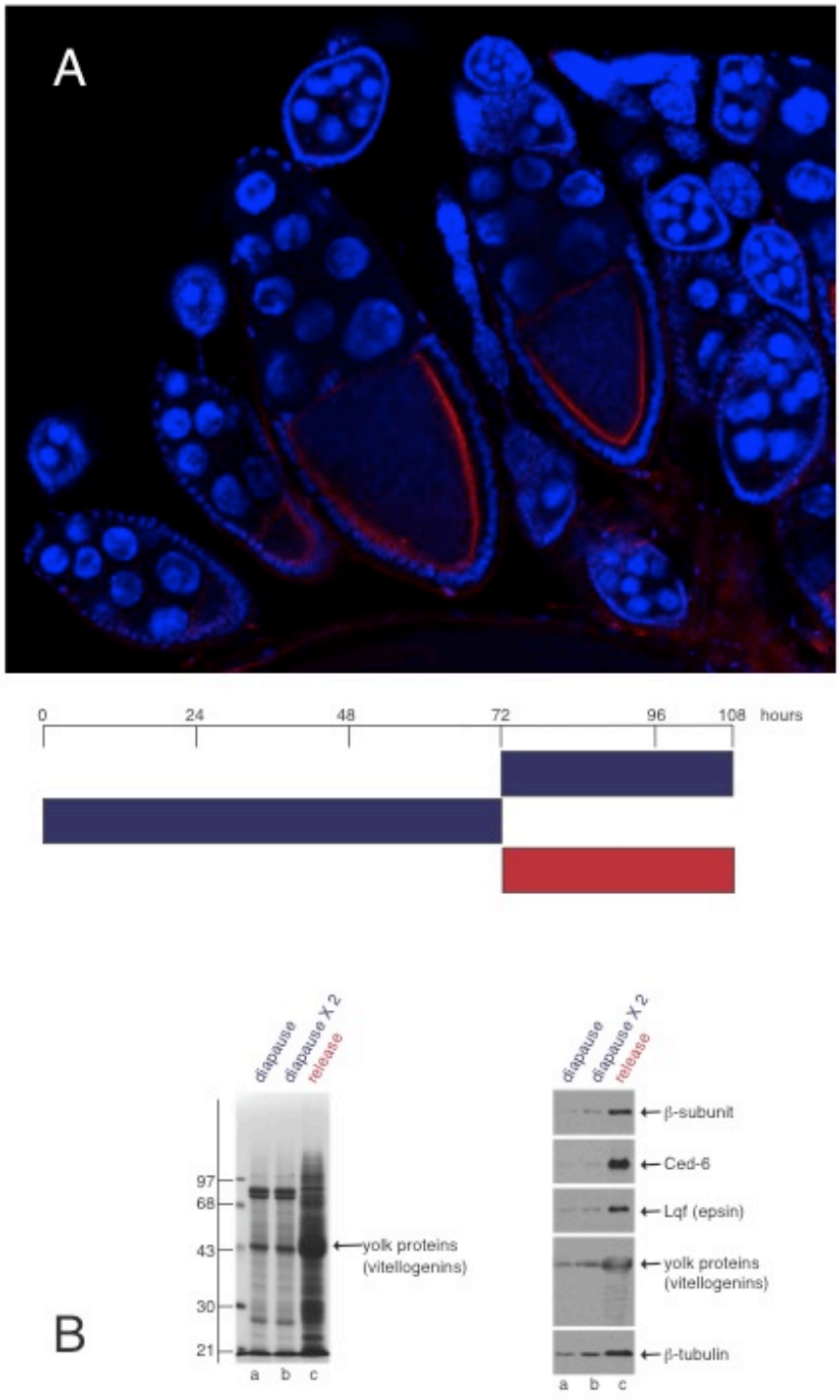


Figure 2.2: Endocytosis in pre-vitellogenic (diapausal and vitellogenic) *Drosophila* ovaries.

(A) mCherry-RAP internalization in pre-vitellogenic and vitellogenic oocyte respectively and (B) Coomassie stained gel (left panel) and western blot (right panel) analysis of pre-vitellogenic (Diapausal, lane a and b) and vitellogenic (normal, lane c) oocyte lysate.

Immunoblotting was performed to observe the abundance of endocytic proteins, and the results suggest that there was an increase of β-adaptin of AP-2 and epsin during yolk protein uptake. At the same time, there was an increase in the expression of the phagocytic protein Ced-6 during vitellogenesis (Figure 2.2 B). This result suggested a possible role of Ced-6 in oogenesis and led us to suspect that Ced-6 could function in oocyte development and vitellogenesis. Moreover, a Treefam (TF314159) search revealed Ced-6 to be the closest PTB-domain-containing protein to trephin, a mosquito ARH ortholog.

2.2.3 Ced-6 is an endocytic protein

Although the above result suggests there was Ced-6 protein expression in ovaries, the localization in the egg chamber remained undetermined. *In situ* hybridization was performed to observe the *ced-6* transcript expression, and the result indicated that *ced-6* transcripts were highly enriched in oocytes (Appendix A.3). To see the expression pattern of Ced-6 protein, whole-mount immunofluorescence was performed. Like *ced-6* transcripts, Ced-6 protein was highly enriched in the oocyte as compared to nurse cells and follicle cells (Figure 2.3 B). Moreover, the localization of Ced-6 became cortical during stages with high endocytic activity (Figure 2.3 B). Ced-6 localization also occurred concurrently with the clathrin cortical relocalization during vitellogenesis (Figure 2.3 C).

Many endocytic adaptor proteins interact with clathrin and AP-2, therefore I biochemically validated the interaction between Ced-6 and clathrin and AP-2. To observe the interaction, full-length Ced-6 was cloned in a bacterial expression vector pGEX-4T-1, and the protein was expressed as a GST-fusion. GST alone was used as

negative control, and GST-ARH (180-308) was used as a positive control. GST-ARH (180-308) has been shown to bind to both clathrin and AP-2 (He et al., 2002; Mishra et al., 2002b). The mammalian homolog of Ced-6 is Gulp, a known phagocytic protein, so GST-Gulp was also used to observe the interaction. A GST pull-down assay using recombinant proteins and rat brain cytosol clearly showed that GST-Ced-6 and GST-ARH (180-308) strongly interacted with clathrin and AP-2 (Figure 2.3 A). GST-Gulp was also shown to interact with AP-2, whereas the interaction with clathrin was found to be weak. The clathrin heavy chain and the large (~100 kDa) AP-2 α and β subunits are the main binding partners as demonstrated using Coomassie-stained gels and immunoblotting with anti-clathrin heavy chain and anti-β1/β2 antibodies (Figure 2.3 A).

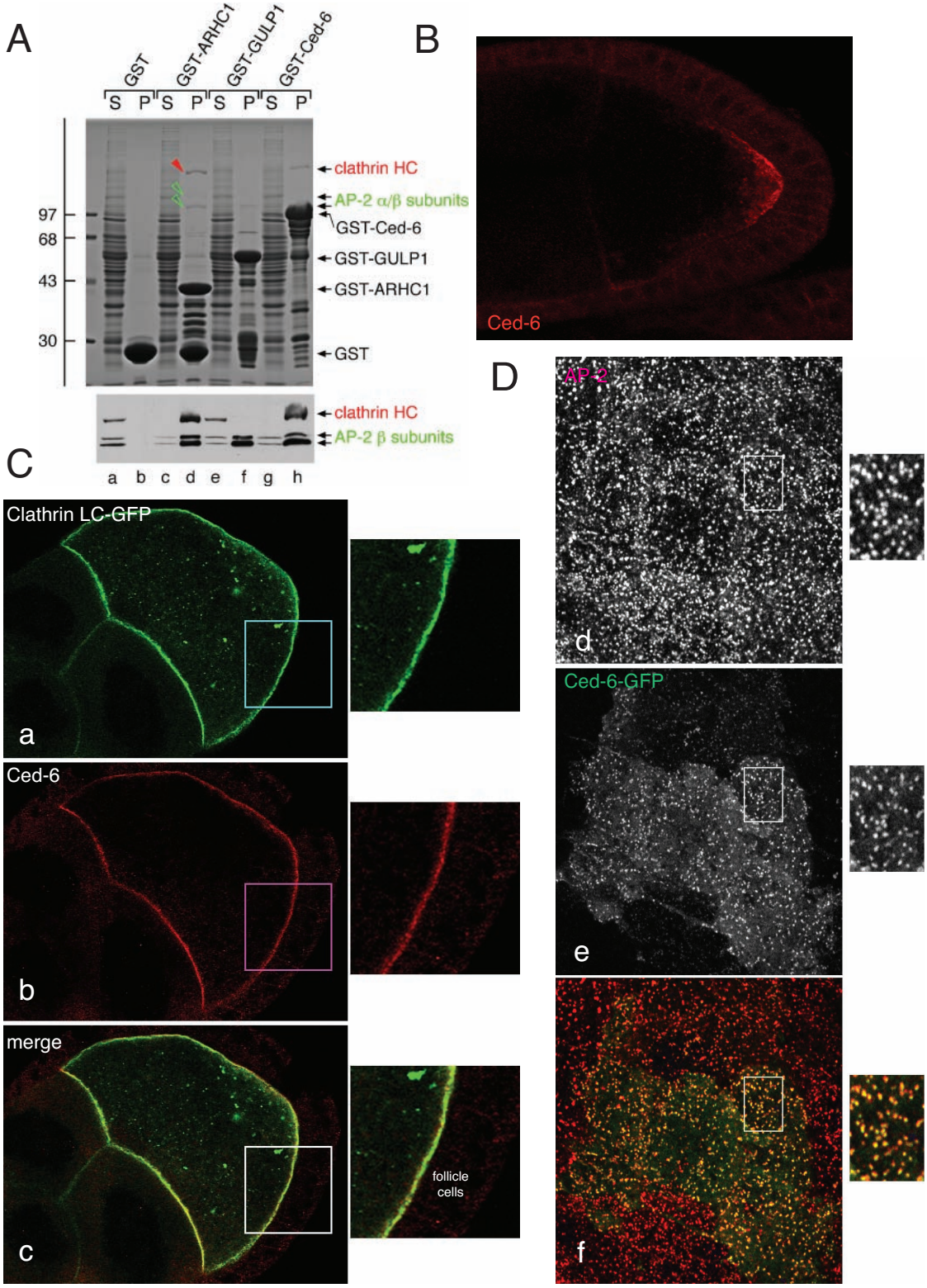

A
GST
GST-ARHC1
GST-GULP1
GST-Ced-6
S P S P S P S P
97
68
43
30
clathrin HC
AP-2 α/β subunits
GST-Ced-6
GST-GULP1
GST-ARHC1
GST
clathrin HC
AP-2 β subunits
a b c d e f g h
B
Ced-6
C
Clathrin LC-GFP
a
Ced-6
b
merge
c
follicle cells
D
AP-2
d
Ced-6-GFP
e
f

Figure 2.3: Ced-6 is an endocytic protein.

(A) GST pull-down assay using 100 μg of GST (lanes a and b), GST-ARHC1 (lanes c and d), GST-Gulp1 (lanes e and f) or GST-Ced-6 (lanes g and h) immobilized on GSH-Sepharose. Sepharose-bound protein was incubated with rat brain cytosol. After centrifugation, aliquots corresponding to 1/40 of each supernatant (*S*) and 1/8 of each washed pellet (*P*) were resolved by SDS-PAGE and either stained with Coomassie Blue or transferred to nitrocellulose. Portions of the blots were probed with the anti-clathrin HC mAb TD.1, anti-AP-1 β1 and AP-2 β2 subunit mAb (1/100). (B) Expression of Ced-6 (red) in a stage 10 oocyte, follicle and nurse cells was determined by immunostaining. (C) Localization of Ced-6 (b) along with GFP-clathrin light chain (CLC) expressing stage 9 oocyte, (a) and merged (panel c). (D) Co-localization of AP-2 (panel d) in Ced-6-GFP expressing (panel e and merged panel f) in HeLa cells.

Drosophila clathrin and AP-2 are highly homologous to their mammalian orthologs and therefore rat brain cytosol containing mammalian AP-2 and clathrin can be used to see the interaction with the *Drosophila* Ced-6 (Bazinet *et al.*, 1993; Camidge and Pearse, 1994; Zhang and Broadie, 1999). GST-Ced-6 and GST-Ced-6 (285-517) were shown to bind to clathrin and AP-2 with equal strength (data not shown). Generally, the unstructured carboxy-termini of endocytic proteins contain the information for clathrin and AP-2 binding in the form of short peptide interaction motifs (Brett *et al.*, 2002). Therefore, the carboxy-terminus of Ced-6 (amino acids 285-517) likely possesses a signal for clathrin binding; a schematic representation of Ced-6 shows putative LLID- and DPF-like interaction sequences (Figure 2.4 A). Different variations of the type I LLDLE sequence are found in endocytic proteins and binds to the clathrin heavy chain (Dell'Angelica, 2001). The DPF sequence has been shown to bind to AP-2 (Benmerah et al., 1996; Owen et al., 1999; Traub et al., 1999b). Ced-6 also contains an AP-2-binding DPF motif positioned at residues 468-500. To evaluate the potential binding properties of these motifs several C-terminal truncations of GST-fused Ced-6 were made. Pull-down assays using purified rat brain cytosol were performed to determine the effects of these

truncations on protein binding. A GST-fused Ced-6 containing only amino acids 401-500 bound to AP-2 as robustly as Ced-6 containing residues 285-517 (Figure 2.4 A [b, lanes c, d, m and n]). This result suggests that, as expected, Ced-6 was able to bind to AP-2 through the DPF sequence.

To further confirm this result, Ced-6 region 401-468 was made by removal of the last 50 amino acids. Removal of the putative AP-2 binding region substantially decreased the binding with AP-2 as well as the binding to clathrin, as seen on the Coomassie blue-stained gel and the immunoblot (Figure 2.4 A [b, lanes k and l]). However, the binding was not completely abolished. This result suggested that Ced-6 contains an additional uncharacterized AP-2 binding region. To further investigate this possibility, the DPF-APA region was mutated in three different contexts. Mutation of DPF-APA did not affect AP-2 binding in GST-Ced-6 (285-517) (Figure 2.4 C, lanes c, d, e and f), but GST-Ced-6 (370-517) showed some decrease in apparent affinity (Figure 2.4 C, lanes g, h, i and j). AP-2 binding was significantly decreased in GST-Ced-6 (401-517) with the DPF-APA mutation (Figure 2.4 C, lanes k, l, m and n); however, clathrin binding remained unaffected. These results validated that the DFP motif in Ced-6 is responsible for AP-2 binding, although Ced-6 also uses one or more other uncharacterized regions for the AP-2 interaction. These experiments also suggested that the Ced-6 C-terminus harbors several clathrin binding regions, since truncations in the C-terminus decreased the clathrin binding and the DPF-APA mutation did not affect it. GST-Ced-6 C1(285-517) interacted with clathrin with similar intensity as GST-Ced-6 (401-517) (Fig 2.4B, lanes c, d and g and h).

Ced-6 (401-517) does not contain any known sequence for clathrin binding based on our knowledge of LLID-type sequences (Dell'Angelica, 2001) (Figure 2.4 A [a]).

Therefore, it was of interest to confirm the region of Ced-6 that is important for clathrin binding, so several regions of Ced-6 C-terminus were tested for interaction with soluble clathrin trimers. These results could not confirm the sequence for clathrin binding, but rather suggested that possibly several regions in the Ced-6 carboxy-terminus are responsible for clathrin binding, as removal of these regions affected clathrin binding. Similarly, AP180 contains multiple clathrin binding sites that are preformed structures dispersed along the expansive clathrin binding region (Zhuo *et al.*, 2010). Overall, these results suggest that clathrin and AP-2 bind independently to Ced-6, since mutation of DPF-APA does not affect clathrin binding, and GST-Ced-6 (401-500) binds to AP-2 robustly, but clathrin binding is significantly reduced (Figure 2.4 A [b, lanes m and n]).

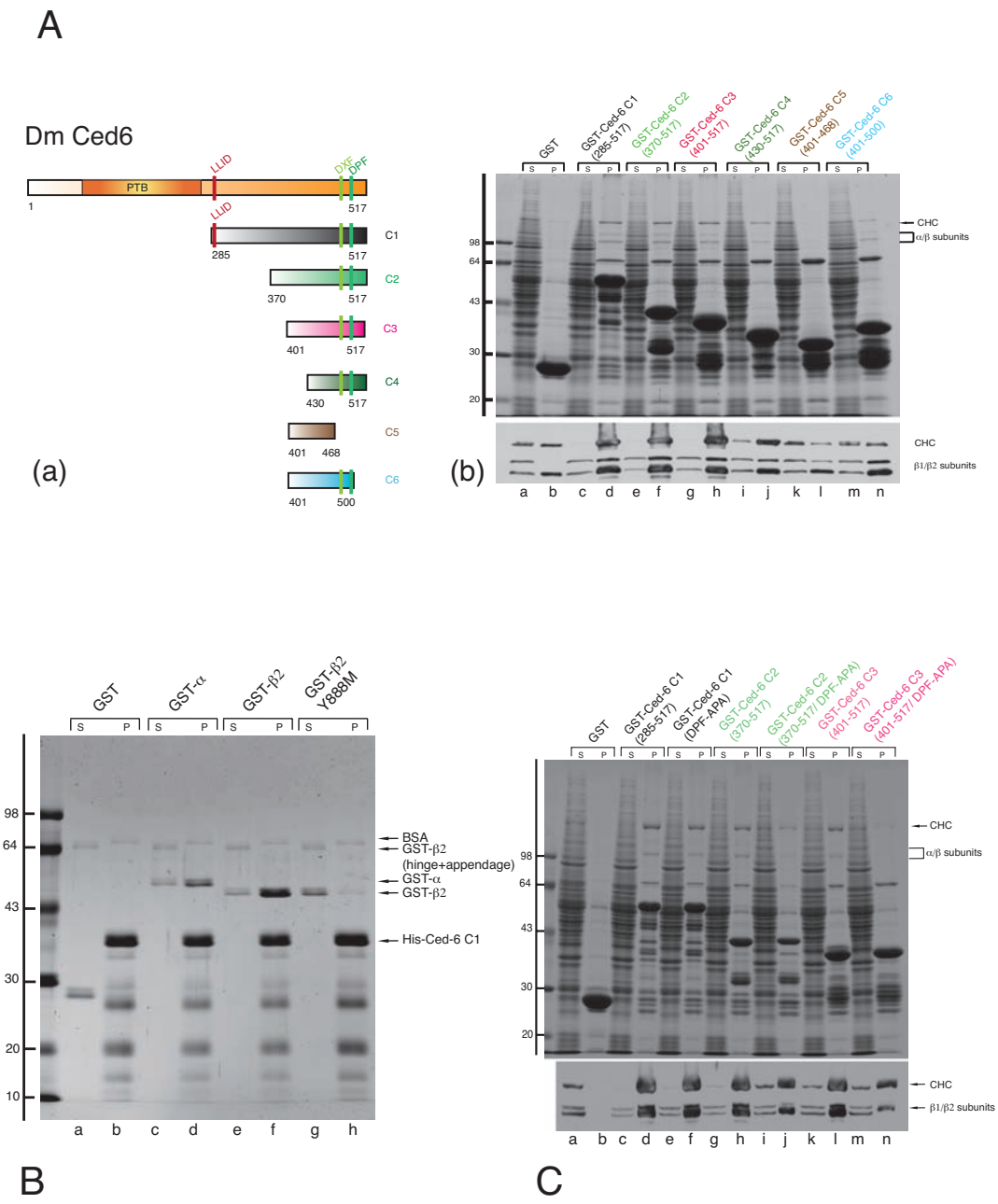

A
Dm Ced6
PTB
LLID
DXF
DPF
1
517
285
370
401
430
468
500
C1
C2
C3
C4
C5
C6
(a)
(b)
GST
GST-Ced-6 C1 (285-517)
GST-Ced-6 C2 (370-517)
GST-Ced-6 C3 (401-517)
GST-Ced-6 C4 (430-517)
GST-Ced-6 C5 (401-468)
GST-Ced-6 C6 (401-500)
S
P
98
64
43
30
20
CHC
α/β subunits
β1/β2 subunits
a b c d e f g h i j k l m n
B
GST
GST-α
GST-β2
GST-β2 Y888M
10
BSA
GST-β2 (hinge+appendage)
GST-α
GST-β2
His-Ced-6 C1
a b c d e f g h
C
GST-Ced-6 C1 (DPF-APA)
GST-Ced-6 C2 (370-517) DPF-APA)
GST-Ced-6 C3 (401-517) DPF-APA)
a b c d e f g h i j k l m n

Figure 2.4: Ced-6 is an endocytic protein.

(A) Schematic illustration of *Drosophila* Ced-6 showing domain structure and different truncation regions. GST pull-down assay using 100 μg of GST (lanes a and b), GST-Ced-6 (285-517) (lanes c and d), GST-Ced-6 (370-517) (lanes e and f), GST-Ced-6 (401-517) (lanes i and j), GST-Ced-6 (401-468) (lanes k and l) or GST-Ced-6 (401-500) (lanes m and n) immobilized on GSH-Sepharose incubated with rat brain cytosol. After centrifugation, aliquots corresponding to 1/40 of each supernatant (*S*) and 1/8 of each washed pellet (*P*) were resolved by SDS-PAGE and either stained with Coomassie Blue or transferred to nitrocellulose. Portions of the blots were probed with the anti-clathrin HC mAb TD.1 and anti-AP-1 β1 and AP-2 β2 subunit mAb (1/100). (B) Approximately 25 μg of His_6-Ced-6*,* immobilized on Ni-NTA-agarose, was incubated with 5 μg of GST (lanes a and b), GST-α appendage (lanes c–d), GST-β2 (lanes e *and* f) or GST-β2(Y888M) (lanes g *and* h*)* in the presence of 0.10 mg/ml BSA. After centrifugation, aliquots corresponding to 1/125 of each supernatant (*S*) and 1/8 of each washed pellet (*P*) were resolved by SDS-PAGE and stained with Coomassie Blue. (C) GST pull-down assay using 100 μg of GST (lanes a and b), GST-Ced-6 (285-517) (lanes c and d), GST-Ced-6 [285-517 (DPF-APA)] (lanes e and f), GST-Ced-6 (370-517) (lanes g and h), GST-Ced-6 [370-517(DPF-APA) (lanes i and j), GST-Ced-6 (401-517) (lanes k and l) or GST-Ced-6 [401-517(DPF-APA) (lanes m and n) immobilized on GSH-Sepharose and incubated with rat brain cytosol. After centrifugation, aliquots corresponding to 1/40 of each supernatant (*S*) and 1/8 of each washed pellet (*P*) were resolved by SDS-PAGE and either stained with Coomassie Blue or transferred to nitrocellulose. Portions of the blots were probed with the anti-clathrin HC mAb TD.1 and anti-AP-1 β1 and AP-2 β2 subunit mAb (1/100).

In binary interaction assays, immobilized His_6-tagged Ced-6 (285-517) was incubated with soluble GST-α appendage and GST-β appendage, and Ced-6 engaged both α and β appendages efficiently. Both of these appendages interact with Ced-6 and thus are recovered in the pellet (Figure 2.4 B, lanes c, d, e and f). Conversely, the β appendage mutation (Y888M) did not bind to the His_6-Ced-6 (285-517) and remained in the supernatant (Figure 2.4 B, lane g) as did GST (Figure 2.4 B, lane a). The structures of the β appendage and α appendage are similar because both have two sub-domains with similar topology (Owen *et al.*, 2000). Both of these appendages of AP-2 can bind to

AP180, epsin and eps15 and therefore are involved in stabilizing the interaction during clathrin-coated pit formation and maturation (Mishra et al., 2005; Traub et al., 1999a). The two proteins contain a partly-conserved binding site of hydrophobic residues on the C-terminal platform sub-domain, indicating that both appendages have the capacity to bind similar motifs such as DϕF/W. The binding-site residues are also conserved in β1-adaptin of AP-1, so this homologue would be predicted to bind the same ligands as β2. Indeed, a GST-Ced-6 construct did bind to β1 as shown in pull-down experiments. All of the experiments just described confirmed that direct interaction of Ced-6 occurs with AP-2 via the DPF motif.

Ced-6 contains clathrin and AP-2 binding sites at its C-terminus. At the N-terminus it contains a PTB domain that is adjacent to a coiled-coil domain. Different concentrations of thrombin-cleaved Ced-6 (1-300) were incubated with either PtdIns(4,5)P_2 containing liposomes or liposomes lacking PtdIns(4,5)P_2. Binding was demonstrated with a Coomassie-stained gel (Figure 2.5 A) and quantified (Figure 2.5 C). A liposome-binding experiment showed that the Ced-6 N-terminus co-precipitated with PtdIns(4,5)P_2-containing membrane with higher affinity than those without PtdIns(4,5)P_2.

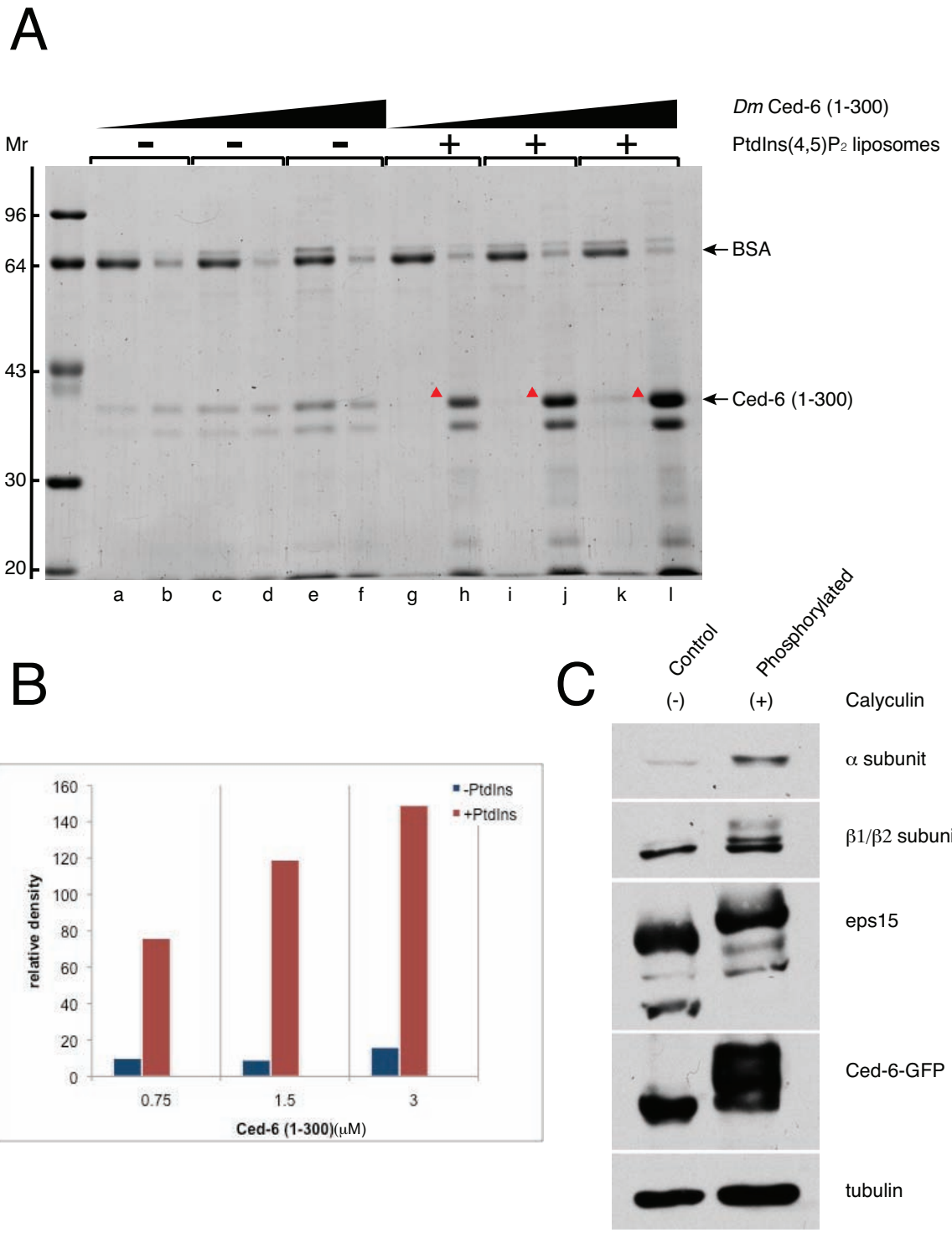

A
Dm Ced-6 (1-300)
PtdIns(4,5)P2 liposomes
Mr
96
64
43
30
20
BSA
Ced-6 (1-300)
a b c d e f g h i j k l
B
-PtdIns
+PtdIns
relative density
160
140
120
100
80
60
40
20
0
0.75
1.5
3
Ced-6 (1-300)(μM)
C
Control
Phosphorylated
(-)
(+)
Calyculin
α subunit
β1/β2 subunit
eps15
Ced-6-GFP
tubulin

Figure 2.5: Ced-6 efficiently associates with the membrane and is phosphorylated.

(A) Thrombin-cleaved Ced-6 PTB domain (1-300) (0.75, 1.5 or 3 μM) was incubated with control liposome alone (lanes a, b, c, d, e and f) or Ptdins(4,5)P_2-containing (Folch fraction) liposomes (lanes g, h, i, j, k and l) in presence of 100 μg/ml BSA. (A) After centrifugation, aliquots of 1/25th of each supernatant (S) or one-fourth of each pellet (P) were analyzed by SDS/PAGE and stained with Coomassie Blue. (B) Relative density of binding affinity of Ced-6 in Coomassie Blue stained gel quantified using Image J. (C) Approximately 25 μg of Ced-6-GFP overexpressing HeLa cell lysates untreated (control) or treated with 100 nM Calyculin A were resolved on SDS-PAGE and transferred onto nitrocellulose membrane for immunoblot. Portions of the blots were probed with anti-α subunit mAb AP.6, anti anti-β1/β2 subunit mAb (1/100), rabbit anti-eps15, rabbit anti-GFP or mAb anti-tubulin antibody.

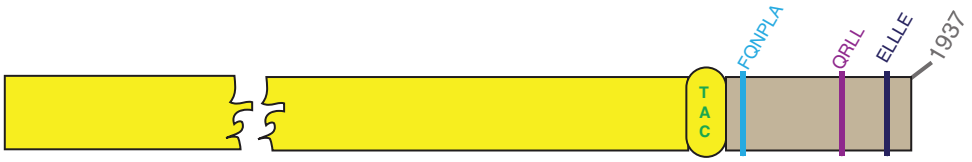
An *in vivo* system was used to further verify the interaction properties of Ced-6. Ced-6 was cloned into mammalian expression vector pEGFPN1 and then ectopically expressed in HeLa SS6 cells. Ectopically expressed Ced-6 showed a punctate distribution with a high co-localization of Ced-6-GFP with AP-2 (Figure 2.3 D) and eps15 (data not shown).

Some of the clathrin-mediated adaptors have been shown to be regulated by phosphorylation (Wilde and Brodsky, 1996). Phosphoproteome analysis of *Drosophila* embryos and *Drosophila* Kc167 cells determined that some residues of Ced-6 are phosphorylated (Bodenmiller *et al.*, 2007; Zhai *et al.*, 2008). This information led to investigation of Ced-6 phosphorylation. HeLa cells were transfected with Ced-6-GFP and the transfected cells were treated with the broad-spectrum phosphatase inhibitor calyculin A, followed by preparation of cell lysates and detection of protein by western blot. As shown in Figure 2.5 C, there was a mobility shift in Ced-6-GFP after calyculin A treatment. The migration pattern of Ced-6-GFP showed a step-like profile, strongly

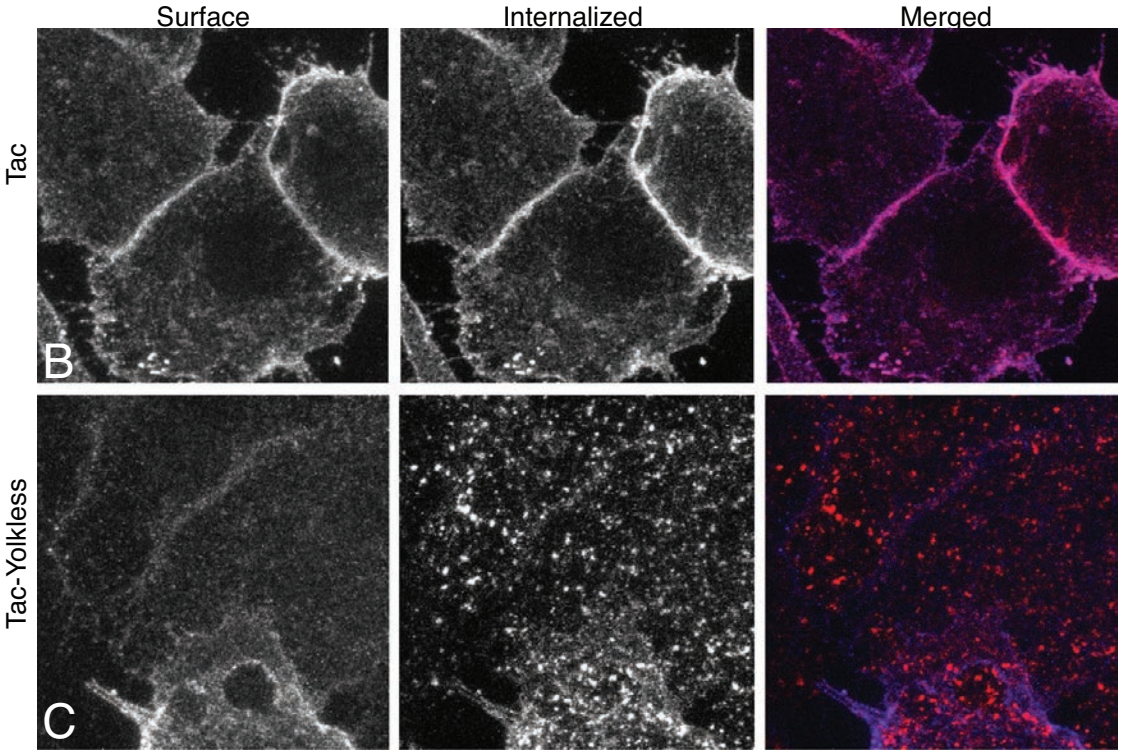
suggesting that several residues were phosphorylated in Ced-6. Known phosphorylated endocytic proteins such as β and α subunit of AP-2 and eps15 further validated this assumption. It is noteworthy that as with other endocytic proteins, evidence of Ced-6-GFP phosphorylation is observed after incubation with calyculin A. This suggests that along with other endocytic proteins only small portion of Ced-6-GFP is in phosphorylated form at steady-state level. Therefore, Ced-6 has several characteristics that make it similar to other clathrin-associated endocytic adaptors. Based on these results, my main questions were: Does Ced-6 play a role in endocytosis? How is Yolkless internalized by clathrin-mediated endocytosis?

2.2.4 Internalization motifs in Yolkless

To understand the mechanism of Yolkless internalization by clathrin, a mammalian expression system using HeLa cells was used. Yolkless is a large protein, with most of its endocytic information contained in its C-terminal cytosolic tail. To study Yolkless endocytosis, the cytosolic domain of isoform A was fused with the extracellular and transmembrane segment of Tac, the α chain of IL2 receptor (CD25) (Uchiyama *et al.*, 1981) (Figure 2.6 A). Internalized Tac was monitored by uptake of mouse anti-Tac antibody raised against extracellular domain. To distinguish the internalized *vs*. surface population, internal Tac was conjugated to the Alexa554 fluorophore, whereas surface Tac was labeled with Cy5 fluorophore. In HeLa cells, Tac was localized to the plasma membrane (Figure 2.6 B), and the Tac-Yl-cytosolic tail (tac-Yl-CT) was efficiently internalized after a 10-minute chase at 37˚C (Figure 2.6 C). This result suggests that cytosolic domain of Yolkless facilitates efficient Tac internalization.



A




```
D. melanogaster   tm-RQRGHTDLNINMHFQNPLATLGGTKAFLEHERAEAGVGFTTETGTVSSRGSND-----TFTTTSAT-SSFAAQQFSVPNALQRLLRPRQSA----------SGDPMAQELLLESPSRES 58
D. yakuba         tm-RKRGHTDLNINMHFQNPLATLSGTKGFMEHERAEAGVGFAPETGTVSSRGSND-----TFTTTTTTSSSFVAQQFSVPNALQRLLRPRQAA----------SGDPMAQELLLESP-RES 54
D. willistoni     tm-RKRGHKDLNINLHFQNPLARLNS-KTIEEAEQQQQ----------QQPGTIG-----YGTSTTTSTSSFVPEQFTRPTLMQRLWPTKQSAVSGTVTATAKYNGEEMVTDILLENT-RGS 49
D. ananassae      tm-RRRGHRDLNINLHFQNPLATLA--RADPKATESSAVVEFAPEQGYGSEEPASQ----------------PPAGMAAPNVFQRFMRSRQS----------RDDPMATELLLDTP-RAS 46
D. seschellia     tm-RQRGHTDLNINMHFQNPLATLGGTKAFLDHERTEAGVGFATETGTVSSRGSND-----TFTTTSAS-SSFAAQQFSVPNALQRLLRPRQSA----------SGDPMAQELLLESP-RES 52
D. erecta         tm-RQRGHTDLNINMHFQNPLATLAGTKGFLEHERAEAGVGFASETGTASSRGSND-----TFTTTSAS-SSFAAQQFSVPNALQRLLRPRQAT----------SGDPMAQELLLESP-RES 54
D. pseudoobscura  tm-RQRGHTDLNINLHFQNPLATIGS-KAFLDHERNEAIVN--GMDGMASSHSSNE-----TGTTSASS--SFAAQQFGMPNVLQRLLRPKQAS----------SGSHMATDMLLENS-RAS 47
D. grimshawi      tm-RQRGHRDLNVTMHFQNPLAMLDG-KSIAEIDHHSE---------DNNSPGIST-----TSTAGYFTPDEGGTNQFGISNILQTLLRRRQAK----------SGGDFTAEVPLENR---- 11
D. mojavensis     tm-RQRGHRDLNINLHFQNPLATLGD-KPLREDGNEVDLVCDSGSSTSSRGISAHT---HTTATGTAPYTAGREPLQFGVPKILQRFLQQRQA----------QSSEMATQVPLENT-RSS 48
D. virilis        tm-RQRGHRDLNINLHFQNPLATLAG-KSIQEIGNEVDLAGEGGGGTSSTSTSTSTSINSSTGTTAASFTAGRETLHFGVPKILQRLLQQRQT----------SNSELVTEVPLENTRQSS 47
```

D

Figure 2.6: Essential internalization motifs of Yolkless.

(A) Schematic illustration of the overall domain organization of Tac-Yolkless (1829-1937) showing possible sorting motifs; it was cloned into the mammalian expression vector pCDNA. (B-C) Ectopic expression of Tac alone (B) or Tac-Yolkless (C) in HeLa cells pulsed with mouse anti-Tac tagged with Alexa594 at 4˚C for 1 hour and chased at 37˚C for 10 min. Fixed and blocked cells were labeled with donkey anti-mouse IgG-Cy5 to visualize surface labeled Tac. (D) Yolkless cytosolic tail alignment in different species of *Drosophila* using ClustalW.

The next set of experiments was designed to determine the protein region and amino acids responsible for efficient internalization of Yolkless. Motif alignment of the cytosolic tail of Yolkless from ten different *Drosophila* species suggested the presence of three highly-conserved regions (Figure 2.6 C). The first identified motif was FxNPxA, and the other two were non-canonical dileucine-type motifs at positions 1901-1902 and 1918-1919 (Figure 2.6 C). To determine which conserved amino acids were responsible for Yolkless endocytosis, three stop codons were introduced into Tac-Yolkless. These different constructs were transfected and chased at 37˚C. The results suggest that all three constructs were expressed efficiently in HeLa cells and delivered to the cell surface. However, only Tac-Yolkless wt and Tac-Yolkless (1829-1919) were internalized along with transferrin, as demonstrated by their presence in the large internal endosomal compartment (Figure 2.7 B). Tac-Yolkless (1829-1884) and Tac-Yolkless (1829-1906) remained at the surface (Figure 2.7 B.). The results of this experiment suggest that the region between 1906 and 1919 of the Yolkless cytosolic tail contains the sequence information necessary for its internalization. Yolkless follows a similar path used by the transferrin receptor for internalization. The endocytic motif of many receptors binds to the heterotetrameric AP-2 complex, and this binding results in the internalization of

receptors (Hirst and Robinson, 1998). The adaptor complex AP-2 can bind to tyrosine and dileucine-based signals (Ohno *et al.*, 1995b).

Tac-Yolkless (1906-1919) contains a non-canonical dileucine-like motif. To gain insight into whether AP-2 is necessary for Yolkless internalization, three different approaches were taken. In the first, mutation in Tac-Yolkless, $LL1^{918\text{-}1919}AA$, was shown to inhibit its internalization, whereas transferrin was internalized efficiently (Figure 2.8 A and B). The next experiments further supported the notion that AP-2 is important for recognizing the $LL^{1918\text{-}1919}AA$ signal and Tac-Yolkless endocytosis. An siRNA-mediated depletion of AP-2 α subunit inhibited transferrin uptake and Tac-Yolkless internalization, whereas in non-depleted cells efficient Tac-Yolkless and transferrin endocytosis occurred (Figure 2.9 E, I, F and H). Knockdown efficiency was confirmed by immunoblotting as well as with immunostaining (Figure 2.9 A, B and C). Similar results were found with clathrin knockdown experiments, further confirming the role of clathrin-mediated endocytosis in Tac-Yolkless uptake (Appendix A.1).

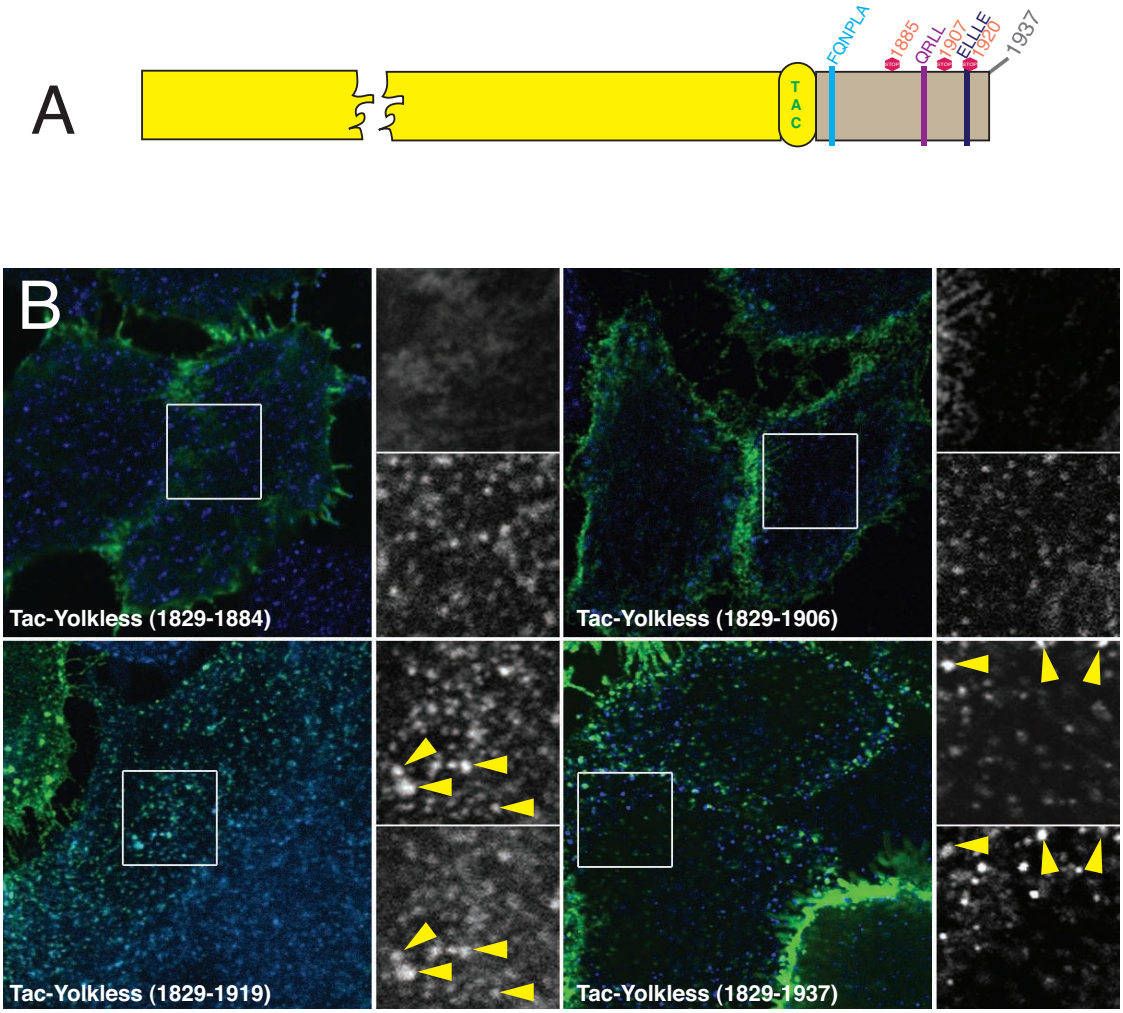
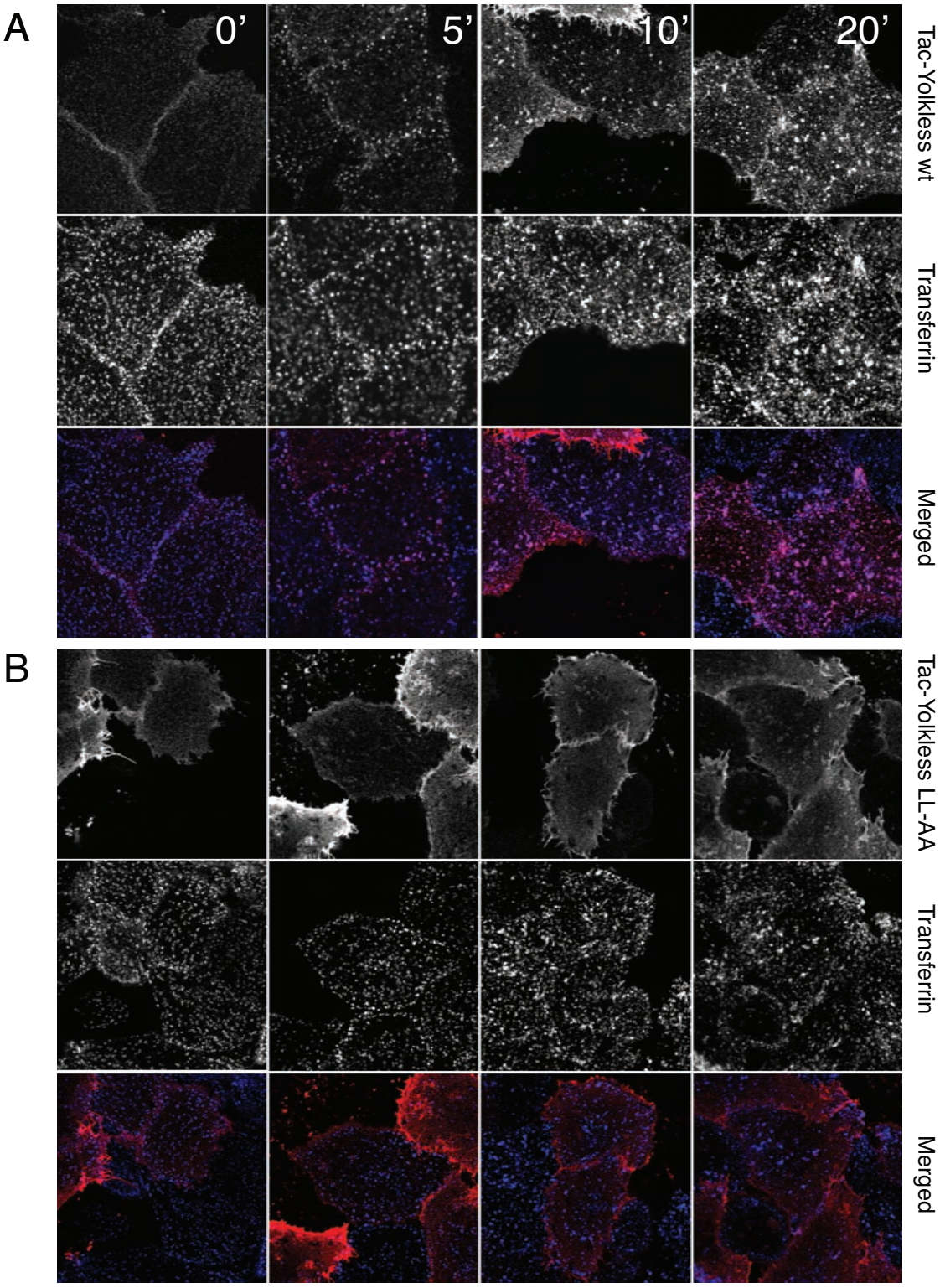


Figure 2.7: Delineation of Yolkless cytosolic tail required for its internalization.

(A) Schematic illustration showing different stop codons (at 1885, 1907 and 1920) introduced in Tac-Yolkless. (B) Ectopic expression of different regions of Tac-Yolkless (1829-1884 and 1829-1906) top panels and Tac-Yolkless (1829-1920 and 1829-1937) bottom panels were starved for 1h in starvation media and pulsed on ice for 1h using mouse anti-Tac antibody and 20 μg/ml transferrin-633 and chased at 37˚C for 10 min. Cells were fixed and permeabilized and probed with donkey anti mouse-IgG-Alexa488 to visualize localization using confocal microscopy; yellow arrowhead in bottom panels shows the co-localization of transferrin633 and Tac-Yolkless.


A
0’
5’
10’
20’
Tac-Yolkless wt
Transferrin
Merged
B
Tac-Yolkless LL-AA
Transferrin
Merged

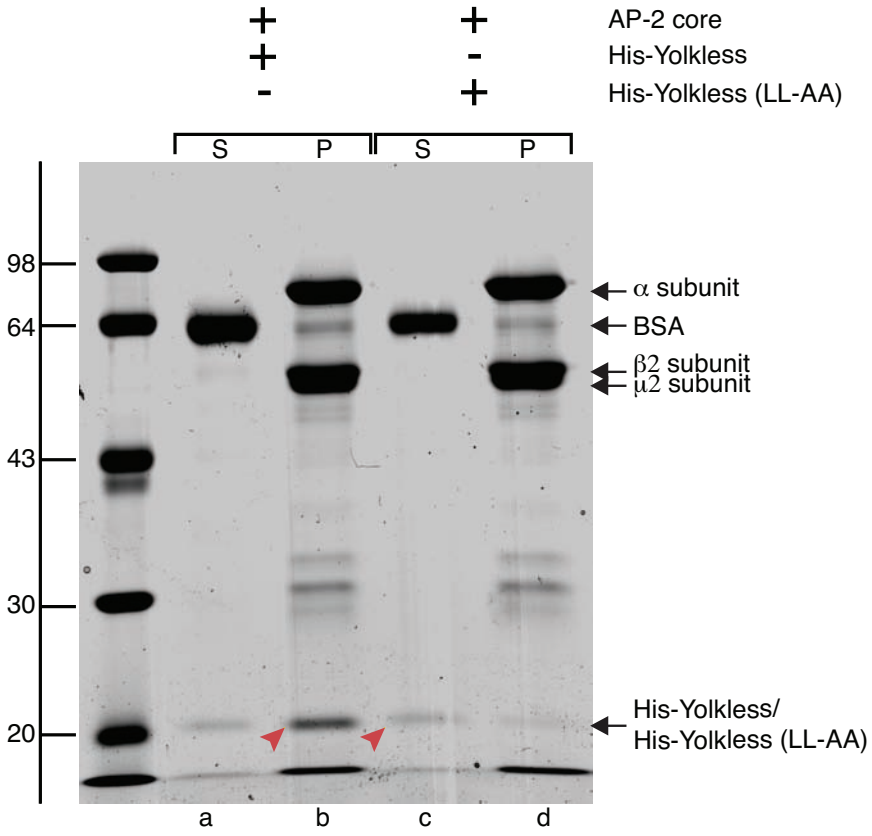


Figure 2.8: Importance of dileucine motifs in Yolkless.

Tac-Yolkless (A) or Tac-Yolkless LL-AA (B) ectopically expressed in HeLa cells were pulsed with mouse anti-Tac and 20 μg transferrin-Alexa-633 at 4°C for 1 hour and chased at 37°C for 0, 5, 10 or 20 min. Fixed and permeabilized cells were labeled with donkey anti-mouse IgG-Cy3 to visualize the internalized Tac. (C) Approximately 10 μg of GST-AP-2 core (lanes a-d) immobilized on GSH-Sepharose was incubated with 4 μg of His6-Yolkless (lanes a and b) or His6-Yolkless-LL-AA (lanes c and d) in the presence of 0.10 mg/ml BSA. After centrifugation, aliquots corresponding to 1/70 of each supernatant (*S*) and 1/6 of each washed pellet (*P*) were resolved by SDS-PAGE and stained with Coomassie Blue.

In the next approach, the His_6-Yolkless cytosolic domain was shown to interact with the recombinant heterotetrameric AP-2 adaptor core, which contains all four subunits of AP-2 but not the appendage domains of α and β (Figure 2.8 C, lanes a and b). The mutation in $LL^{1918-1919}AA$, which inhibited Tac-Yolkless endocytosis, also

diminished His-Yolkless binding to purified AP-2 core (Figure 2.8 C, lanes c and d). These data suggest that non-typical dileucine motif efficiently engages AP-2 and results in endocytosis by clathrin. However, a recent report suggests that AP-2 α or σ2 subunit mutant germline clones efficiently accumulate yolk protein in the oocyte (Parra-Peralbo and Culi, 2011). A previous study has shown that *yolkless* mutant oocytes display a 90% decrease in clathrin-coated structure (DiMario and Mahowald, 1987). This information led us to think that there are one or more proteins that can function as clathrin-mediated endocytic adaptors along with AP-2. Previously published results by several groups have suggested the role of functionally redundant CLASPs in the absence of AP-2 (Conner and Schmid, 2002; Motley et al., 2003). To understand what the other mechanism for interaction could be, the cytosolic region of Yolkless sequence was carefully examined. The cytosolic regions of Yolkless receptor for ten different *Drosophila* species were shown to contain a highly conserved FxNPxA sequence. First, it was of interest to determine whether the highly-conserved FxNPxA sequence could function as an endocytic sequence. The above experimental results suggested that the Tac-Yolkless (1829-1884) construct containing only FxNPxA sorting signal was expressed efficiently in HeLa cells but was not internalized alone or along with transferrin. Similarly, Tac-Yolkless harboring the $LL^{1918-1919}AA$ mutation was also found only on the cell surface while transferrin was internalized normally. These results suggest that the FxNPxA motif was non-functional for Yolkless internalization in HeLa cells. Previously, it was found that changing the FxNPxY to FxNPxA abrogates the internalization of LDLR cytosolic tail (Chen *et al.*, 1990). To study the FxNPxA sequence, a reverse mutation was created with FxNPxA mutated to FxNPxY. This change in Tac-Yolkless resulted in clustering with transferrin at 0˚C without chase (Figure 2.10 E), while Tac-Yolkless FxNPxA-

FxNPxY and LL$^{1918\text{-}19}$AA was efficiently internalized at 37˚C after the chase (Figure 2.10 F). This data confirmed the idea that FxNPxA can be recognized by a PTB domain CLASP and can function as a sorting signal in cytosolic tail of Yolkless.

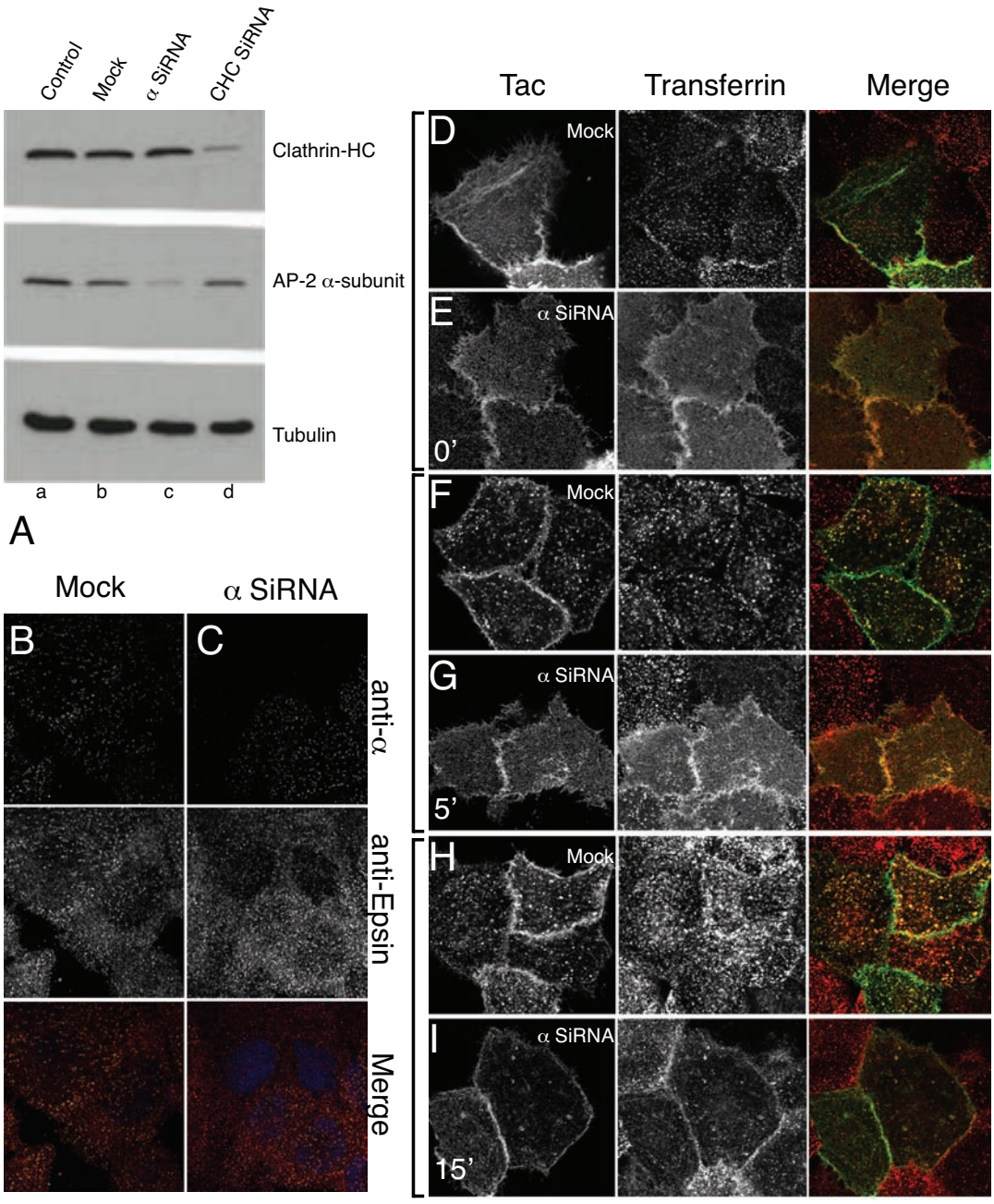


Figure 2.9: Importance of AP-2 in Tac-Yolkless endocytosis.

(A) HeLa cell lysates (25 μg) of control, mock, AP-2 α siRNA treated or CHC siRNA treated cells were resolved on SDS-PAGE and transferred to nitrocellulose. Portions of the blots were probed with clathrin heavy chain anti-TD.1 mAb, AP-2 anti-α subunit mAb or anti-tubulin mAb antibody. Fixed and permeabilized Hells cells transfected with mock (panel B) or AP-2 α siRNA (panel C) were incubated with AP-2 anti-AP.6 mAb (green) or rabbit anti-epsin (red) to visualize on confocal microscope. Ectopic expression of Tac-Yolkless in mock (panels D, F and H) or AP-2 α siRNA (panels E, G and I) were starved for 1 h and pulsed on ice for 1 h using mouse anti-Tac antibody and 20 μg/ml transferrin-568 and chased at 37°C for 0, 5 or 15 min. Cells were fixed and permeabilized and probed with donkey anti-mouse-Alexa488 to visualize. Representative single optical sections are shown.

HeLa cells express Dab2 and ARH, and both function redundantly in the internalization of LDLR (Keyel et al., 2006a). In next experiment, I wanted to confirm whether ectopic expression of ARH-GFP could help in internalization of Tac-Yolkless (1829-1884), which contains only FxNPxA motif. The high concentration of exogenously expressed Tac-Yolkless might be a hindrance in its uptake by endogenous PTB domain proteins. The result shows that even after 5 minutes of chase, Tac-Yolkless (1829-1884) remained at the surface while transferrin internalized normally (Figure 2.11 E). Even though FxNPxA can function as an endocytic signal, ARH-GFP is unable to bind to the FxNPxA motif and thus unable to function as an adaptor for Tac-Yolkless (Figure 2.11 D, E and F).

Since Ced-6 was present in oocytes and possesses several essential features consistent with properties of known CLASPs, I decided to validate the function of Ced-6 in Yolkless endocytosis. First, I sought to determine whether both Ced-6-GFP and Tac-Yolkless are co-localized and present in clathrin-coated pits. Indeed, when Ced-6-GFP was co-expressed with Yolkless in HeLa cells, Ced-6-GFP clustered with Yolkless in the

coated pit and was co-localized with transferrin (Figure 2.12 A). To determine whether the PTB domain of Ced-6-GFP could participate in the internalization of Tac-Yolkless (1829-1884), Ced-6-GFP was co-expressed with Tac-Yolkless (1829-1884) or with Tac-Yolkless $LL^{1918-1919}AA$ mutation in HeLa cells, with the knowledge that neither of these proteins could be internalized by themselves, as they possess only the FxNPxA motif. Surprisingly, the presence of Ced-6-GFP clustered these two Tac-Yolkless constructs in clathrin-coated pits along with transferrin at 0˚C without chase, which suggests that Ced-6-GFP interacted with Tac-Yolkless (1829-1884) and Tac-Yolkless $LL^{1918-1919}AA$ just as the wild-type Yolkless does (Figure 2.12 C). In separate experiments, Tac-Yolkless (1829-1884) alone or along with Ced-6-GFP was transfected into HeLa cells and chased for 10 minutes at 37°C. Tac conjugated with Alexa594 was used to detect the internalized population of Tac and Cy5 was used to detect the surface population (Figure 2.12 B).

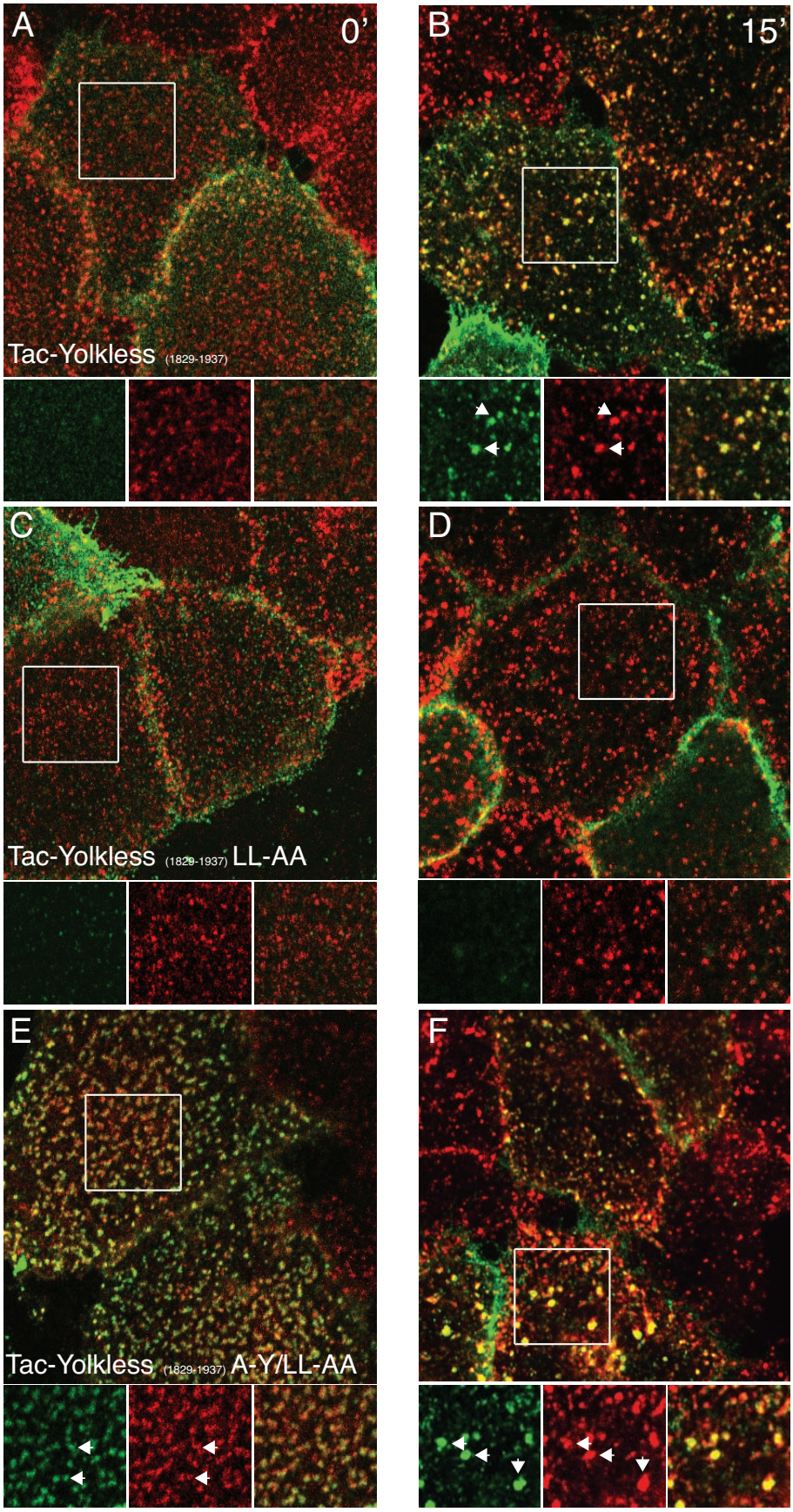

A
0’
Tac-Yolkless (1829-1937)
B
15’
C
Tac-Yolkless (1829-1937) LL-AA
D
E
Tac-Yolkless (1829-1937) A-Y/LL-AA
F

Figure 2.10: Importance of FxNPxA motif in Yolkless internalization.

HeLa cells transiently transfected with (A and B) Tac-Yl (1829-1937), (C and D) Tac-Yl (1829-1937) LL-AA or Tac-Yl (1829-1937) A-Y/LL-AA (E and F) were serum starved for 1 h at 37°C and incubated with 4 μg/ml anti-Tac mAb along with 20 μg/ml transferrin-Alexa568 on ice for 1 h without a chase (panels A, C and E) or with a 15-min chase at 37°C (panels B, D and F). Cells were fixed and saponin permeabilized. Color-separated single representative optical images are shown. White arrowhead indicates the co-localization of Tac (green) with transferrin (red).

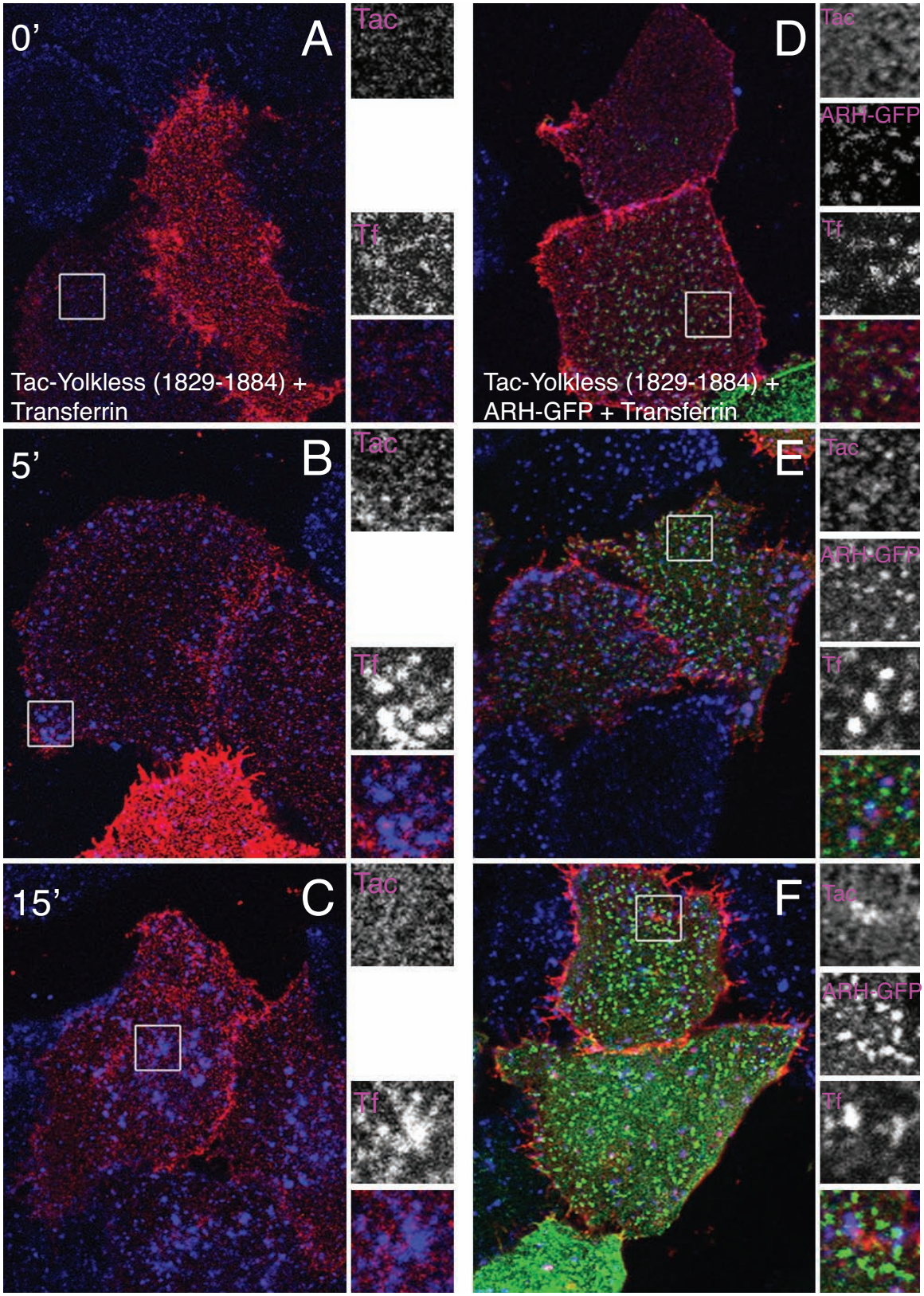
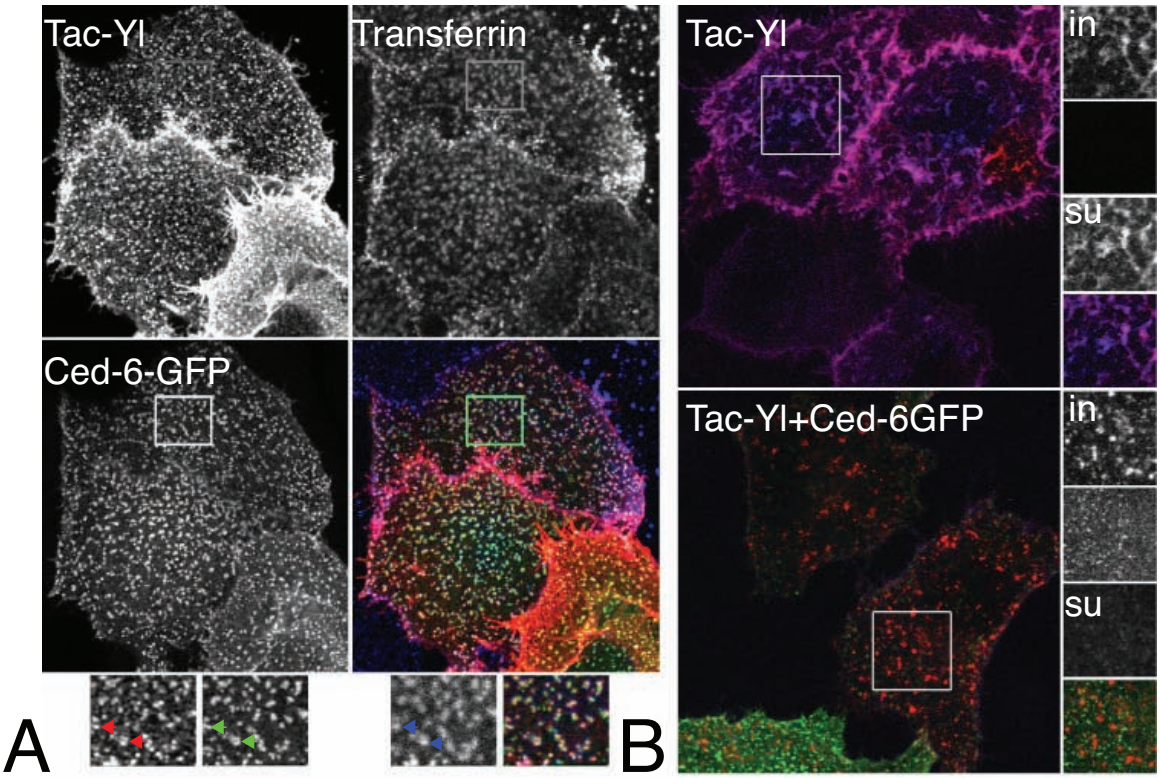

0’
A
Tac
Tf
Tac-Yolkless (1829-1884) +
Transferrin
D
Tac
ARH-GFP
Tf
Tac-Yolkless (1829-1884) +
ARH-GFP + Transferrin
5’
B
Tac
Tf
E
Tac
ARH-GFP
Tf
15’
C
Tac
Tf
F
Tac
ARH-GFP
Tf

Figure 2.11: Overexpression of ARH-GFP and Yolkless internalization.

(A, B and C) HeLa cells transiently transfected with Tac-Yl (1829-1884 containing only FxNPxA) and (D, E and F) HeLa cells transiently transfected with Tac-Yl (1829-1884) and ARH-GFP were serum starved for 1 h at 37°C and incubated with 4 μg/ml anti-Tac mAb along with 20 μg/ml transferrin-633 on ice for 1 h without a chase (panels A and D), with a 5-min chase at 37˚C (panels B and E) or with a 15-min chase at 37˚C (panels C and F). Cells were fixed and saponin permeabilized. Color-separated single representative optical images are shown.


Tac-Yl
Transferrin
Ced-6-GFP
A
Tac-Yl
in
su
Tac-Yl+Ced-6GFP
in
su
B

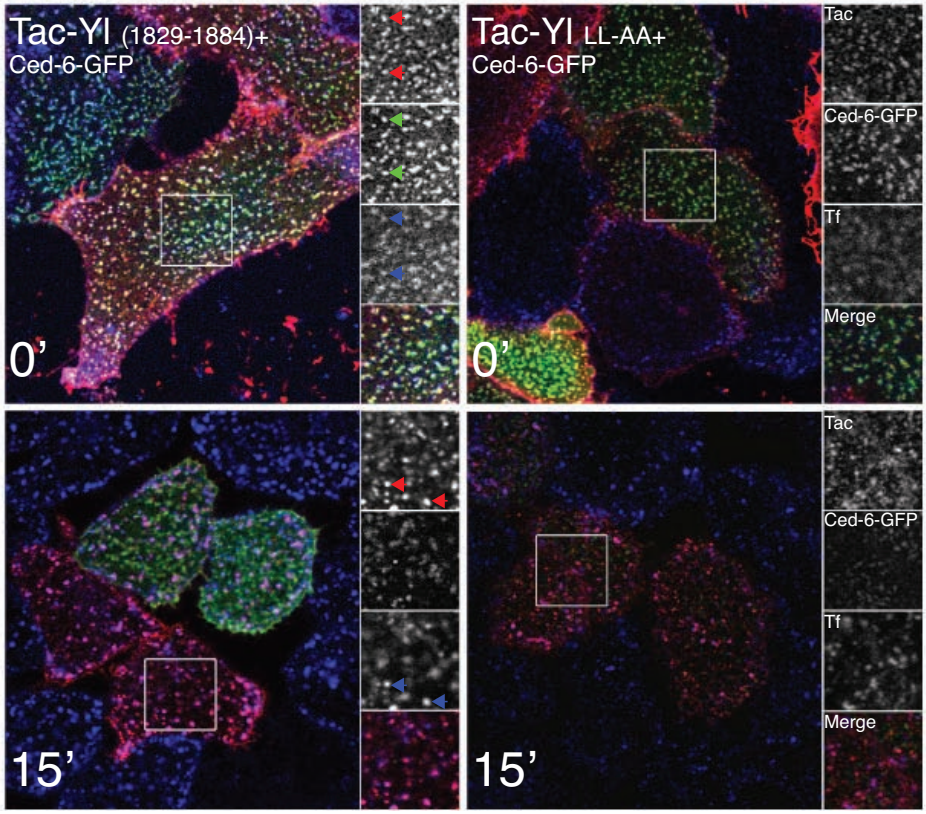

Tac-Yl (1829-1884)+
Ced-6-GFP
0'
Tac-Yl LL-AA+
Ced-6-GFP
0'
Tac
Ced-6-GFP
Tf
Merge
15'
15'
Tac
Ced-6-GFP
Tf
Merge
C

Figure 2.12: Requirement of Ced-6 in Yolkless internalization.

(A) HeLa cells transiently co-expressing Tac-Yl and Ced-6-GFP were serum starved for 1 h at 37°C, followed by incubation with 4 μg/ml anti-Tac mAb and 20 μg/ml transferrin-633 on ice. Fixed and permeabilized cells were incubated with anti-mouse IgG-Cy3. Representative single optical images in the box area represent Tac-Yl co-localized with Ced-6-GFP and transferrin. (B) Surface and internalized pool of Tac in HeLa cells transiently transfected with Tac-Yl (1829-1884) alone (top panel) or with Tac-Yl (1829-1884) along with Ced-6-GFP (bottom panels). Cells were serum starved for 1 h at 37°C, and cells were incubated with 4 μg/ml anti-Tac mAb tagged with Alexa594 on ice for 1 h. Cells were chased for 10 min at 37°C. Fixed and blocked cells were incubated with anti-mouse IgG-Cy5 to visualize the surface population. Representative single optical images in the box area represent Tac-Yl (1829-1884) internalization. (C) HeLa cells transiently transfected with Tac-Yl (1829-1884) or Tac-Yl (1829-1937) LL-AA along with Ced-6-GFP were serum starved for 1 h at 37°C, followed by incubation with 4 μg/ml anti-Tac mAb and 20 μg/ml transferrin-633 on ice. Cells were pulsed at 37°C for 0 or 15 minutes. Fixed and saponin permeabilized cells were incubated with anti-mouse IgG-Cy3. Representative single optical images in the box area represent Tac-Yl (1829-1884) and LL-AA mutation co-localized with Ced-6-GFP at 0 min. At 15 min Tac and transferrin were internalized.

The next experiment was to further confirm the role of PTB domain of Ced-6 in Tac-Yolkless internalization. PTB-domain-containing mammalian proteins such as Dab2 and ARH can bind to receptors with the FxNPxY sequence and can also engage the membrane (Keyel et al., 2006a). Based on previous studies, Ced-6-GFP was mutated to S164T and F233V assuming that these mutations would disrupt the CED-6 PTB domain binding to its ligand (Figure 2.13). First it was confirmed that these mutations did not alter the localization of Ced-6 to the clathrin-coat (Figure 2.13 A and B). Next Ced-6-GFP (S164T and F233V) was co-expressed along with Tac-Yolkless (1829-1884); the results suggest that whereas wild-type Ced-6-GFP clusters Tac-Yolkless into clathrin-coated pits and helps in its internalization (Figure 2.13 F and G), Ced-6-GFP (S164T and

F233V) neither clusters Tac-Yolkless (1829-1884) nor internalizes Tac-Yolkless (1829-1884) (Figure 2.13 H and I).

These results suggest a significant role of Ced-6 in yolk protein endocytosis, and led to experiments to determine the yolk protein accumulation in the oocyte in the absence of Ced-6. To this end, *ced-6*-null flies were used (Kuraishi *et al.*, 2009). The *Drosophila* stock *w; ced-6^{J26}/CyO, Act-GFP* was used to generate the *ced-6* null *Drosophila*. The chromosome with wild-type Ced-6 gene also contains the *CyO, Act-GFP*, which acts as balancer chromosome in this *Drosophila* stock. The flies heterozygous for the *ced-6-null* mutation express GFP and have a curly wing phenotype. Ovary lysates from Canton-S (wild type) and *ced-6* null flies were used to determine the presence of Ced-6, GFP and the β subunit of AP-2. *ced-6* null flies blacked the expression of GFP and Ced-6 but expressed AP-2 at similar levels as wild-type flies (Appendix A.2). These *ced-6*-null flies efficiently accumulated yolk protein and could lay eggs (data not shown).

My endocytic uptake results suggested that Yolkless contains two different motifs for its internalization, and these motifs can act redundantly to ensure Yolkless internalization and yolk protein accumulation. Data presented in this study not only help distinguished certain signals present in the Yolkless cytosolic tail, but also revealed a novel property of Ced-6 in clathrin-mediated endocytosis. Ced-6 is a well-known phagocytic protein that, like the clathrin-mediated endocytic adaptor protein-2 (AP-2), participates in Yolkless endocytosis.

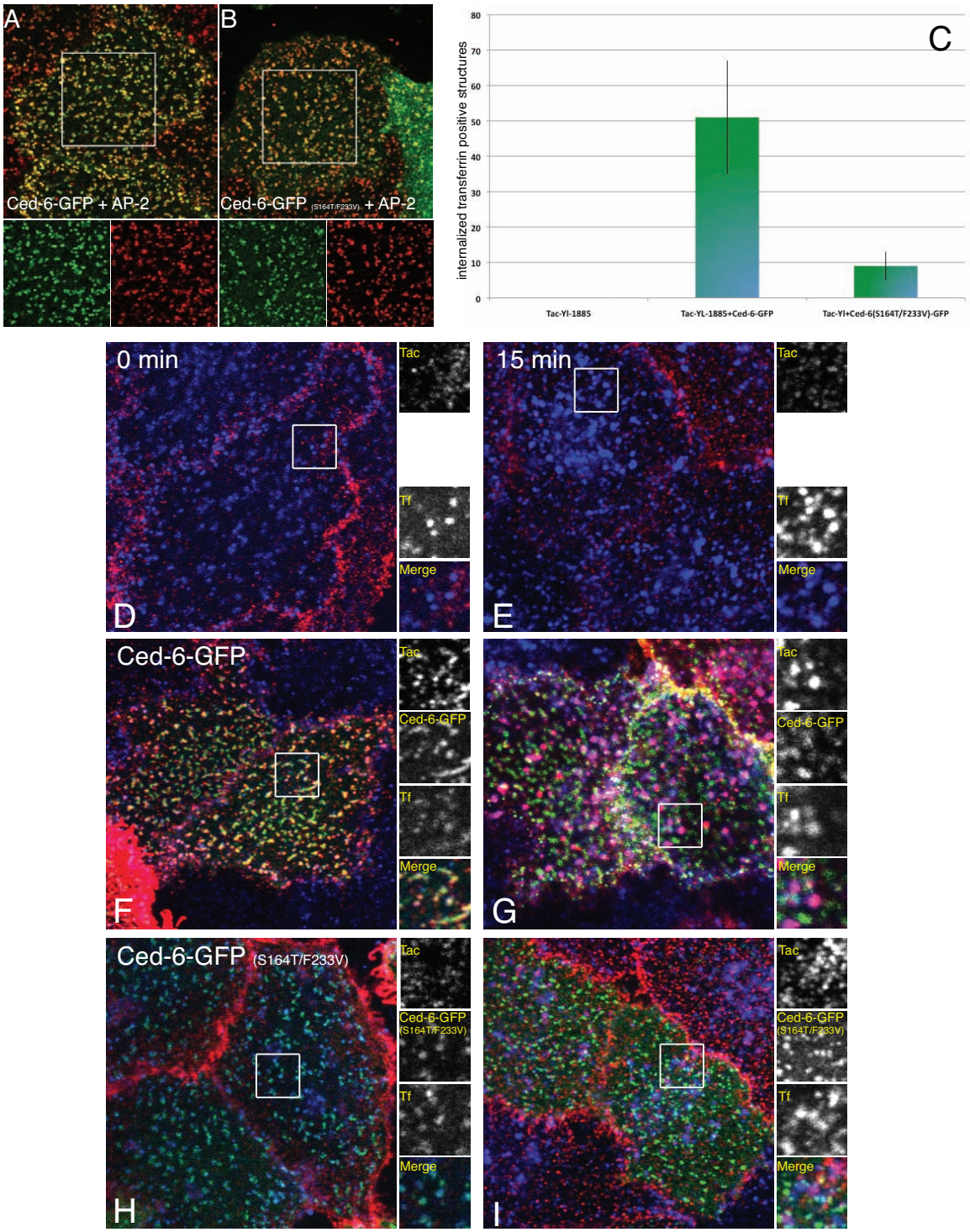

A
Ced-6-GFP + AP-2
B
Ced-6-GFP (S164T/F233V) + AP-2
C
internalized transferrin positive structures
80
70
60
50
40
30
20
10
0
Tac-YI-1885
Tac-YL-1885+Ced-6-GFP
Tac-YI+Ced-6(S164T/F233V)-GFP
0 min
Tac
Tf
Merge
D
15 min
Tac
Tf
Merge
E
Ced-6-GFP
Tac
Ced-6-GFP
Tf
Merge
F
Tac
Ced-6-GFP
Tf
Merge
G
Ced-6-GFP (S164T/F233V)
Tac
Ced-6-GFP (S164T/F233V)
Tf
Merge
H
Tac
Ced-6-GFP (S164T/F233V)
Tf
Merge
I

Figure 2.13: Requirement of the Ced-6 PTB domain in Yolkless internalization.

HeLa cells were transiently transfected with (A) Ced-6-GFP or (B) Ced-6 (S164T/F233V)-GFP. Fixed and saponin permeabilized cells were incubated with AP-2 anti-AP.6 mAb (red). Single representative optical images confirm the steady state distribution of Ced-6 wt and Ced-6 mutant to AP-2 positive compartments. (D and E) HeLa cells were transiently transfected with Tac-Yl (1829-1884) alone, (F and G) with Tac-Yl (1829-1884) and Ced-6-GFP or (H and I) with Tac-Yl (1829-1884) and Ced-6 (S164T/F233V)-GFP. Cells were serum starved for 1 h at 37°C, followed by incubation with 4 μg/ml anti-Tac mAb and 20 μg/ml transferrin-633 on ice. Cells were fixed directly (panels D, F and H) or chased at 37˚C for 15 min before fixation. Cells were saponin permeabilized and incubated with anti-mouse IgG-Cy3. Representative single optical images in the box area represent the internalized Tac-Yl (1829-1884) co-localized with transferrin and Ced-6-GFP; Ced-6 (S164T/F233V)-GFP impaired the ability of Tac-Yl (1829-1884) to internalize as shown in the bar diagram in panel C (n = 25).

2.3 Discussion

In this study, it was found that Ced-6 has all the properties consistent with an endocytic protein, and that it functions as an endocytic adaptor for Yolkless endocytosis in the clathrin-dependent pathway. Yolkless is a receptor required for yolk protein accumulation in oocytes and is required not only for reproduction of *Drosophila* but also for clathrin-coat formation on the oocyte plasma membrane (DiMario and Mahowald, 1987), as *yolkless*-null *Drosophila* oocytes show a 90% reduction in clathrin-coated structures. This evidence suggests that Yolkless is the main receptor internalized by endocytic clathrin-coated structures in the *Drosophila* oocyte. Recent studies have suggested that the Lipophorin receptor, which also belongs to the LDLR superfamily, is present in the *Drosophila* ovary, but that lipid uptake mediated by Lipophorin receptor is independent of endocytosis (Parra-Peralbo and Culi, 2011). However, in same study, yolk internalization was found to be dependent on endocytosis, because *Rab5*-null oocytes

could not accumulate yolk protein. Rab-5 is known to play an important role in the early endocytic pathway (Singer-Kruger *et al.*, 1994).

I identified a non-canonical dileucine-like signal, which can bind to AP-2 and helps in the endocytosis of Yolkless, in this study. Several lines of evidence suggest a functional role for AP-2 in vitellogenic oocytes. Immunoreactivity of the α subunit of AP-2 is cortical in the vittellogenic oocyte (Dollar *et al.*, 2002), and electron microscopic studies show that AP-2 is present in clathrin-coated structures in oocytes (Sommer *et al.*, 2005). Moreover, in a transgenic *Drosophila* line expressing human transferrin, human transferrin is internalized normally in oocytes. The transferrin cytosolic tail contains tyrosine-based Yxxϕ-type sorting signal recognized by AP-2 (Ohno *et al.*, 1995a). This internalization of ectopically expressed receptor in the egg chamber is dependent on dynamin (Bretscher, 1996).

Somewhat unexpectedly, yolk protein efficiently accumulates in the oocyte even in the absence of functional AP-2 (Parra-Peralbo and Culi, 2011). This finding could have two different explanations: One suggests that the absence of AP-2 adaptor protein is compensated for some other endocytic adaptor, which might be present in the clathrin-coated structure; the other hypothesis that Yolkless is internalized by a clathrin-independent pathway. Previously published results by several labs have suggested the role of functionally redundant CLASPs in the absence of AP-2 (Conner and Schmid, 2003; Motley et al., 2003). It was suggested that AP-2 is not an essential component for clathrin-coated pit formation; however, these CLASPs cannot replace AP-2 function completely as AP-2 depletion causes a 12-fold decrease in clathrin-coated pit formation (Motley et al., 2003).

Drosophila AP-2 α subunit expression as shown by analysis of transcript levels and antibody staining suggests a high enrichment in some regions of *Drosophila* embryo and larva (Gonzalez-Gaitan and Jackle, 1997). In that study several mutations of *Drosophila* α adaptin of AP-2 were studied, and it was found that these mutations did not hamper the morphological requirement of neuron biogenesis as *Drosophila* embryos could grow to the larval stage. Therefore, specific defects and localization to certain areas of the embryo suggest that α adaptin is not essential for all clathrin-mediated endocytosis or the maternal expression of α adaptin is sufficient for clathrin-mediated endocytosis until the larval stage of development. In another study, α adaptin mutations that deleted or modified the C-terminal domain of the α subunit, were not lethal to *Drosophila* (Berdnik *et al.*, 2002). These mutations affected the bristle formation, and thus it was concluded that α adaptin is required for several endocytic interactions in clathrin-mediated endocytosis and specifically for Numb.

In the studies done by Parra-Peralbo and Culi (2011) to observe yolk protein endocytosis, they used an α adaptin and σ subunit of *ap-2* mutant germline clones. AP-2-$\alpha^{40\text{-}31}$ ends at amino acid 510 in the trunk region before the hinge and appendage domains and thus is missing the region that interacts with several endocytic accessory domains (Windler and Bilder, 2010). Yolk protein accumulation is also inhibited in *P[SUPor-P]AP-2*σ^{KG024} (Bellen *et al.*, 2004), which contains an insertion in σ subunit of AP-2 (Parra-Peralbo and Culi, 2011).

Adaptins from different AP complexes generally do not show a redundant function. For example, in yeast, overexpression of σ2 does not substitute for the loss of σ1 (Phan *et al.*, 1994). Similarly, the medium subunits of other AP complex proteins cannot substitute for μ2 in *C. elegans* (Gu *et al.*, 2008). In addition, that study further

confirms that AP-2 structure is destabilized in the absence of μ2, suggesting that formation of AP-2 complex in *C. elegans* is highly dependent on μ2, and that μ2 cannot be replaced by another medium subunit AP-complex. All four subunits are important for stabilization of the AP-2 core, as expression of any single AP-2 subunit in bacteria yields an insoluble protein; only simultaneous expression of all four subunits results in a soluble protein complex (Collins et al., 2002a). Therefore, all four subunits of AP-2 are essential for its function as a heterotetramer and depletion or mutation in any subunit can hamper its function.

It is mentioned in several studies that an insertion in the protein coding region does not completely abolish expression of the protein, and the AP-2 expression levels have not been studied after different mutations. Therefore, whether or not AP-2-mediated clathrin-dependent endocytosis of Yolk protein is decreased or completely abolished upon mutations in the AP-2 subunit, requires further study. Several studies have suggested a role for alternative adaptors that not only promote coat assembly, but also drive clathrin-mediated endocytosis in the absence AP-2 (Keyel et al., 2006a; Motley et al., 2003). I have ascertained that one alternative adaptor is Ced-6, which is expressed in oocytes and can bind to both clathrin and AP-2. The N-terminal PTB domain of Ced-6 can recognize the Yolkless cytosolic tail, which contains the unusual sorting signals FxNPxA.

One reason for different sorting signals within a receptor cytosolic tail is that it could promote more rapid internalization than a single signal. The LDL receptor related protein 1 (LRP1) contains both FxNPxY and Yxxϕ signals (Li *et al.*, 2000). The kinetics of LRP1 uptake are faster than that of the LDL receptor, which contains only a single FxNPxY signal for internalization. The inactivation of the Yxxϕ signal in LRP1 decreases the rate of its internalization to that of the LDL receptor (Li et al., 2000). From stages 8-

11 of oogenesis, which are the vitellogenic stages, the oocyte grows rapidly and accumulates vast quantities of yolk protein. This vitellogenic stage lasts for only ~15 hours. Computational modeling to understand the process of receptor-mediated endocytosis has suggested that ligand internalization depends either on the degree of ligand binding or on consumption (Shankaran *et al.*, 2007). This model suggests that *Xenopus* oocytes express a high level of receptors at the surface, and these bind to ligands with high specificity. The accumulation of yolk protein is thus dependent on the internalization rate of receptors (Shankaran et al., 2007). Therefore, multiple sorting signals that can bind to multiple adaptor proteins are required for proper and expedient Yolkless endocytosis. The non-canonical FxNPxA and dileucine signals of Yolkless can interact with Ced-6 and AP-2, respectively. This interaction facilitates rapid Yolkless internalization, and therefore sufficient yolk accumulation in the limited time of oogenesis.

AP-2 and Ced-6 may have low micromolar binding affinity to Yolkless, and this may have functional relevance. AP-2 and Ced-6 form a complex with Yolkless in yolk protein endocytosis (Figure 2.16). Furthermore, the formation and dissociation of these complexes has to be orchestrated with the efficient and rapid endocytosis. It is therefore possible that a very high affinity for Ced-6 and/or AP-2 would trap Yolkless in a particular complex, and thus inhibit it from being quickly recycled back and recruited onto the oocyte membrane for next cycle of endocytosis.

The Ced-6 PTB domain can recognize FxNPxY (Awasaki *et al.*, 2006) and FxNPxA signals (the present study). The cytosolic domain of amyloid precursor protein (APP) has been found to interact with the PTB domain of X11. X11, which also favors the non-phosphotyrosine ligand in the PTB domain binding pocket, contains several basic

amino acid residues that are also present in the other PTB domain containing proteins (Zhang et al., 1997c), but binding of APP to X11 is not affected by mutation of tyrosine to alanine (Borg *et al.*, 1996). Similarly, in a NAK peptide, Phe at 0 position does not make any significant contact with the Numb PTB domain (Zwahlen et al., 2000). In fact, Dab-like PTB-domain binding to ligand is not affected by the residue at 0 position of ligand, but is dependent on hydrophobic interactions and hydrogen bonds between ligand and PTB domain (Uhlik et al., 2005). In most cases the amino acid residue at +1 position makes an important contribution to the binding energy between ligand and PTB domain (Uhlik et al., 2005). Therefore, it could be possible that, like APP binding to X11, Yolkless uses a longer region for PTB domain binding, as three amino acid residues at the N-terminus and one amino acid at the C-terminus are also phylogenetically conserved (Figure 2.6 D). Several studies have shown that in many cases, a disordered region of a protein becomes structured only after interaction with a ligand (Huber and Weis, 2001). Disordered proteins have the intrinsic advantage being able to bind to more than one type of target molecule (Dyson and Wright, 2005). It is anticipated that like X11 PTB domain, the *Drosophila* Ced-6 PTB domain also contains an unstructured region for ligand binding. This unstructured region could help in FxNPxA binding.

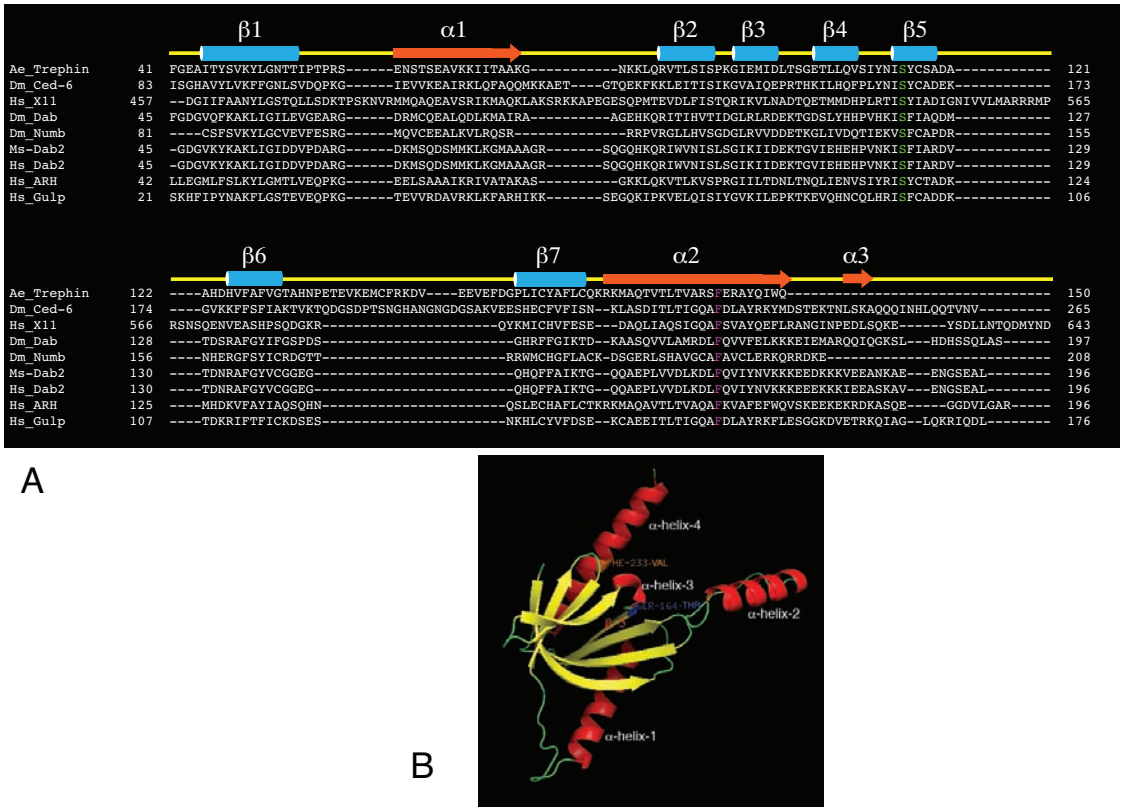


Figure 2.14: PTB domain structural similarity across the species.

(A) PTB domain alignment based on the predicted α-helix (red arrow at the top of the alignment) and the β-sheet in blue cylinders in different species, including *Drosophila melanogaster* (Dm) CED-6 (NM_165676), *Aedes aegypti* (Ae) trephin (XM001662581), *Homo sapiens* (Hs) X11 (NM_00116), *Drosophila* Dab (NM_079395), *Drosophila* Numb (NM_078799), mouse (Ms) Dab2 (NM_023118), human Dab2 (NM_001244871), human ARH (NM_015627) and human Gulp (NM_016315), showing two conserved residues serine (S in green) in β5 and phenylalanine (F in magenta) in the α2 helix (predicted by PromalS3D, http://prodata.swmed.edu/promals3d). (B) Ribbon structure of CED-6 PTB domain predicted from the known structure showing Ser 164 in the β sheet and Phe 233 in the α helix. Structure predicted using swissmodel.expasy.org (http://swissmodel.expasy.org/workspace/) and modified on Pymol.

The X11 PTB domain includes a unique insertion of 20 residues in the β5- β6 loop as compared with ARH and Dab-2. This large loop is positioned near the ligand binding region of X11. Amino acids in this region are mostly disordered in the structure, (Zhang *et al.*, 1997a). Similarly, Ced-6 has an insertion in the β6-β7 loop, and this region could be analogously responsible for diverse peptide interactions (Figure 2.14 A). In this way, the sequence difference between Ced-6 and the ARH and Dab2 PTB domains could account for the capacity of Ced-6 to specifically engage the FxNPxA signal in Yolkless.

The Ced-6 C-terminus contains several residues that can be phosphorylated (Bodenmiller *et al.*, 2007; Zhai *et al.*, 2008). Like several endocytic adaptors, phosphorylation of Numb is important for its localization in clathrin-coated vesicles (Nishimura and Kaibuchi, 2007). Clathrin-mediated endocytosis is tightly regulated by cycles of phosphorylation. Therefore reversible phosphorylation might also regulate the Ced-6 activity during endocytosis by regulating its presence and residency in the clathrin-coated pit.

Ced-6 is implicated in phagocytosis; however, the *Drosophila* oocyte does not show any evidence of engulfment of nurse cells or follicle cells. In fact, dying nurse cells are engulfed by follicle cells, which are epithelial cells, but can show features of phagocytic cells (Cummings and King, 1970). Moreover, immunofluorescence indicates that Draper, a *Drosophila* receptor for phagocytosis, is not expressed in oocytes but is expressed in follicle cells (Figure 3.1). In eukaryotes, clathrin is expressed in both germ cells and somatic cells. Genetic studies with *Drosophila* have demonstrated that different functions of clathrin are genetically discernible (Bazinet *et al.*, 1993). It is possible that clathrin has different functions in various tissues, suggesting a need for specific

regulation of *chc* gene expression during the development of a multicellular organism. In mosquitoes, two different clathrin transcripts are expressed: One is present in somatic cells, whereas the other is expressed in oocytes (germ cells). Expression of clathrin depends on developmental and hormonal signals (Kokoza *et al.*, 1997). Ced-6 expression and function is also regulated in *Drosophila* oocytes as Ced-6 transcript is upregulated in vitellogenic egg chamber (Appendix A.3). Overall, the results from this study suggest a clear role of Ced-6 in clathrin-mediated endocytosis during yolk uptake. I also found that AP-2 physically interacts with Yolkless. We propose a model in which AP-2 and Ced-6 interact synergistically with Yolkless and concentrate Yolkless in coated pits for internalization (Figure 2.15)

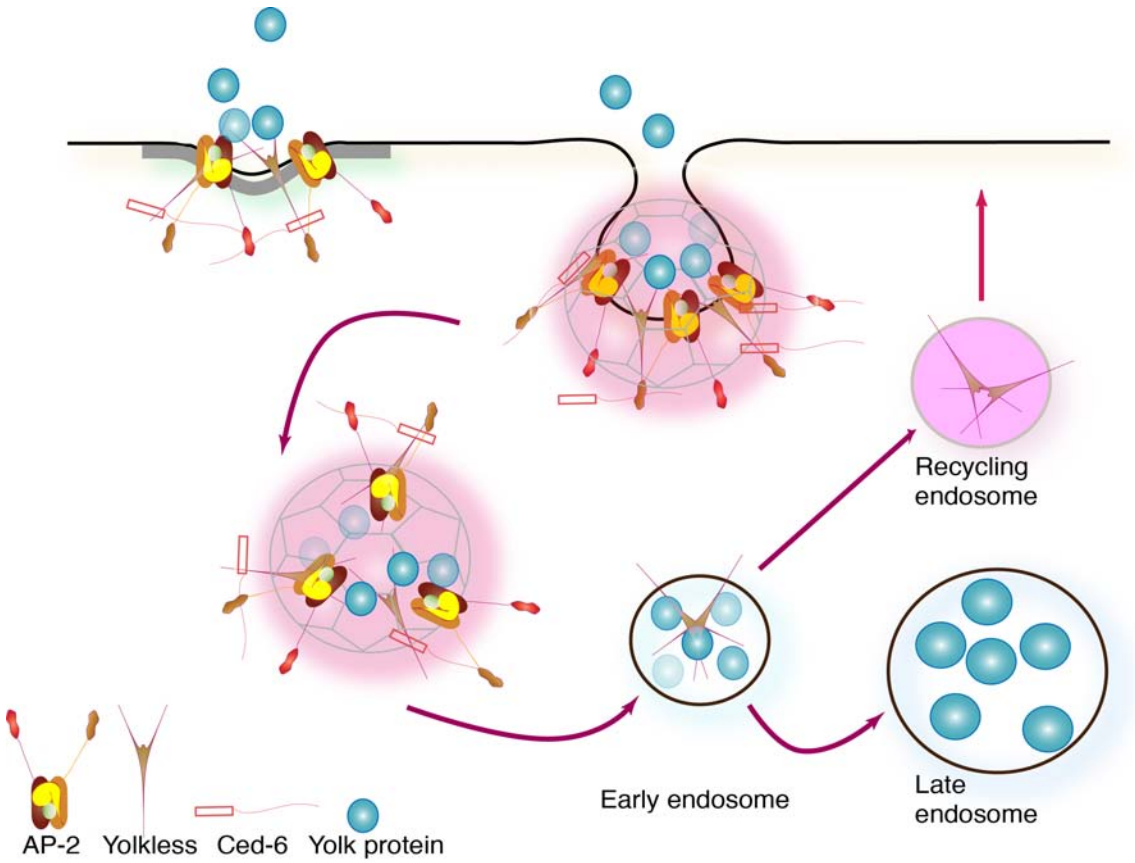


Figure 2.15: Schematic representation of Yolkless endocytosis.

Clathrin-mediated endocytosis of Yolkless occurs with the help of adaptor protein-2 complex and Ced-6. Accumulation of yolk protein is shown in the late endosomal compartment. Yolkless then recycles back to the surface for efficient transport of yolk proteins.

3.0 CONCLUSIONS AND FUTURE DIRECTIONS

3.1 Conclusions

Clathrin-mediated endocytosis was the first-identified and is the most thoroughly described form of endocytosis. Every year new proteins are discovered that function in clathrin-mediated endocytosis. Many proteins have similar binding affinities for clathrin or AP-2 or both, and hence may be important in the endocytic process. Detailed studies of these endocytic proteins have important significance, as new insights into the molecular interactions and mechanisms of these interactions in clathrin-mediated endocytosis will further our understanding of nutrient, receptor and macromolecule internalization. These processes are all critical during development and their dysfunction may lead to pathology. It is well established that within several protein sorting signals such as Yxxϕ, FxNPxY, dileucine and ubiquitin contain the information that results in internalization by clathrin-mediated endocytosis. It has become widely accepted that information present in these sorting signals can be easily distinguished by cognate endocytic sorting proteins. In recent years, it has become appreciated that some plasticity in primary sequence motifs can be accommodated by the endocytic sorting machinery, depending on tissue specificity or organism type. The necessity and biological advantage of this plasticity is analyzed below.

During my thesis work, I tried to untangle an intellectual knot by studying the trafficking of Yolkless. In my dissertation research, I have dissected the pathway of yolk protein internalization by clathrin-mediated endocytosis. Yolk protein, which is essential for *Drosophila* nutrition and development, is internalized by the Yolkless receptor. My findings demonstrate that Ced-6, a well-known phagocytic receptor binding protein,

assists in Yolkless endocytosis. I have demonstrated that the *Drosophila* Ced-6 is present in the egg chamber. Experimental results suggest that along with other clathrin-associated proteins, the Ced-6 levels increase during oocyte development. I have determined that Ced-6 displays all the core properties typically shared by clathrin-associated sorting proteins (CLASPs); this is one of the novel findings of my research. My dissertation describes the role of Ced-6, biochemically and functionally, in endocytosis.

My data show that Ced-6 can interact with clathrin and AP-2. This finding is valuable because until recently, Ced-6 and its homologs have been implicated in phagocytosis (Awasaki *et al.*, 2006). This finding was further strengthened by results of the uptake assay, in which Ced-6 displayed characteristics of a dedicated sorting adaptor for the Yolkless receptor. This study also demonstrated that Ced-6 can interact with Yolkless and efficiently cluster the receptors in clathrin-coated pits. This clustering of Yolkless by Ced-6 results in internalization of Yolkless along with transferrin receptors in HeLa cells. Yolkless contains an FxNPxA sequence that can interact with the PTB domain of Ced-6, and my data suggest that disruption of the Ced-6 PTB domain ligand-binding property inhibits clustering and subsequent endocytosis of Yolkless. I have also demonstrated that the Yolkless cytosolic tail contains a non-canonical dileucine motif. This AP-2-binding motif assists in Yolkless internalization.

Previous studies supported the notion that the Ced-6 PTB domain can interact with the FxNPxY signal of Draper. Draper is also a transmembrane receptor and is involved in cell engulfment by phagocytosis. The importance of the tyrosine side chain in FxNPxY sorting signal has been demonstrated by several groups (Chen *et al.*, 1990; Pedersen *et al.*, 2010). The cytosolic domain of amyloid precursor protein (APP) has been found to interact with PTB domain of X11. Binding of APP is not affected by mutation of

tyrosine to alanine (Borg *et al.*, 1996). The crystal structure of the X11 PTB domain suggests that this PTB domain interacts with amino acid residues that are dispersed across a relatively extended region of the ligand, and there is an insertion of 20 amino acid residues in the PTB domain (Zhang et al., 1997b). In some PTB domains, the binding energy is not dependent on the contacts with the Tyr residue but on hydrophobic interactions and hydrogen bonds between the ligand and PTB domain. The Ced-6 PTB domain might also function analogously to X11 and engage Yolkless in similar way.

3.2 Future directions

A main conclusion drawn from my study is that two well-studied endocytic processes, clathrin-mediated endocytosis and phagocytosis, share some common early steps. Accordingly some important questions have been raised by this study. How does Ced-6 function mechanistically in two different uptake processes? Is there a mechanistic link between phagocytosis and clathrin-mediated endocytosis? In my opinion there is a strong possibility that clathrin-mediated endocytosis and phagocytosis are interconnected. One recent study suggested that absence of the retromer complex protein affects the engulfment and plasma membrane expression of the *C. elegans* CED-1 receptor analogous to expression of fly Draper (Chen *et al.*, 2010). The same study found that the retromer complex helps recycle the CED-1 receptor to the plasma membrane. Similarly, in *Drosophila* glial cells, knockdown of Ced-6 affects Draper expression around damaged axons (Doherty *et al.*, 2009). That study also found that expression of *shibire*^{ts} (a dynamin mutant) in ensheathing glial cells inhibited recruitment of Draper on severed axons and clearance of degenerating axons. They concluded that Ced-6 helps Draper in recruitment of glial membrane around the severed neurons (Doherty *et al.*, 2009). Studies in *C.*

elegans suggested that CED-1, CED-6 and CED-7 directly or indirectly affect actin assembly (Kinchen *et al.*, 2005). So far, studies done on Ced-6 from different organisms clearly indicate that the binding of Ced-6 with receptors occurs at the plasma membrane (Awasaki *et al.*, 2006; Freeman *et al.*, 2003; Su *et al.*, 2002). However, these studies failed to explain the downstream function of Ced-6, so we are still unable to fully understand the mechanism of Ced-6 function. My study sheds some new light on the mechanism of Ced-6 function. It showed that Ced-6 binding to the Yolkless receptor results in accumulation of receptor within clathrin-coated pits. My study also demonstrated, for the first time, that the downstream partners of Ced-6 are endocytic components, and this puts Ced-6 into a new framework. However, to further dissect the role of Ced-6 in clathrin-mediated endocytosis and phagocytosis, it will be necessary to study further the role of Ced-6 in phagocytosis.

3.2.1 Allies of Ced-6

Yolkless is highly expressed in oocytes and its expression is limited to oocytes. Draper is not present in the oocyte, but Draper is expressed in follicle cells (Figure 3.1). Yolkless and Draper could perform distinct processes (i.e., phagocytosis and clathrin-mediated endocytosis) and still use the same PTB domain CLASP. Separate or redundant recognition could be examined experimentally. For example, if Draper is ectopically overexpressed in the germ cell lineage, or more specifically in oocytes, then a similar or distinct interaction mode of Ced-6 with Draper and Yolkless could be discerned. The Draper pathway requires Undertaker, a *Drosophila* junctophilin protein, for phagocytosis (Cuttell *et al.*, 2008).

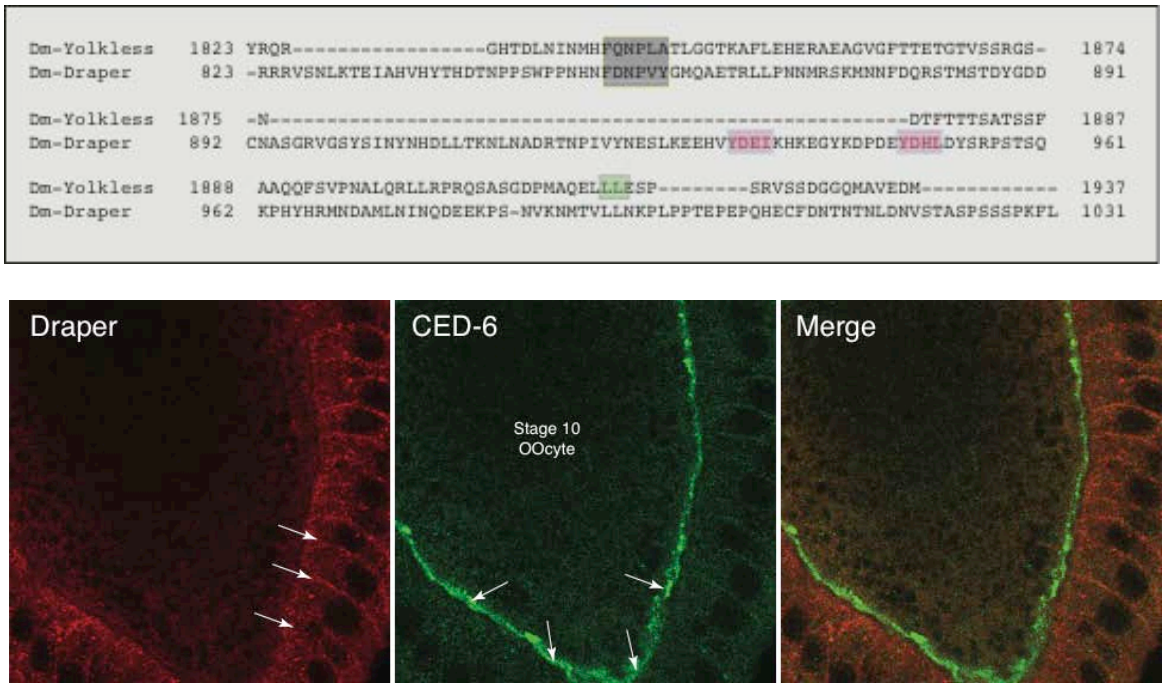


Figure 3.1: *Drosophila* engulfment receptor Draper and Ced-6 expression in the egg chamber.

Top panel: Sequence alignment of *Drosophila* Yolkless showing the NPxA and LL sorting signals and Draper showing the NPxY (in gray box) and YxxI and YxxL (in pink) type sorting signals (top panel). Bottom panel: Confocal image of a stage 10 *Drosophila* ovary showing Draper (indicated by white arrows) in follicle cells (red) and Ced-6 (green) in the cortical region of oocyte. Also shown is the merge image.

Junctophilin is required to form a junctional complex between the plasma membrane and the endoplasmic reticulum and therefore is required for crosstalk between Ca^{2+} channels in the plasma membrane and the endoplasmic reticulum (Takeshima *et al.*, 2000). Ca^{2+} homeostasis is necessary for the Draper pathway in phagocytosis and is most likely detected by Ced-7, an ABC transporter (Cuttell *et al.*, 2008). In *C. elegans* the absence of CED-7 affects the recruitment of CED-1 receptor around the phagocytic cup (Zhou *et al.*, 2001). A *draper* null allele ($drpr\Delta^5$) is embryonic lethal and two

hypomorphic alleles were able to grow to the larval stage (Freeman *et al.*, 2003). However, the *ced-6* null allele is completely viable, suggesting that Draper depends on a different pathway than the well-studied Ced-1/Ced-6/Ced-7 pathway.

The cytosolic tail of FcγR receptor contains an immunoreceptor tyrosine activation motif (ITAM) consisting of tandem YxxI/L motifs. The YxxI/L motif is a site for phosphorylation by a tyrosine src kinase (Flannagan *et al.*, 2011). The Draper cytosolic tail contains the motif YxxILI/L-X_{6-12}-YxxL, which has been shown to directly interact with the tyrosine kinase Shark. This interaction occurs only in the presence of Src42A kinase (Ziegenfuss *et al.*, 2008). That study therefore provides evidence of a second pathway, which may or may not be dependent on Ced-6. Studies done on Ced-6 have associated it with actin recruitment, but Draper can interact with a tyrosine kinase (Ziegenfuss *et al.*, 2008), and Syk tyrosine kinase has been found to induce the signal that further mediates actin assembly (Cox *et al.*, 1996). Therefore, it is likely that actin assembly does not depend on Ced-6 binding to Draper.

The tyrosine residues of Fc gamma receptor III can be phosphorylated and interact with Syk kinase, and the mutation to inhibit tyrosine phosphorylation affects the signaling. The inhibition of phosphorylation does not affect receptor internalization (Bonnerot *et al.*, 1998). Two tyrosine residues present in the ITAM motif of Fc gamma receptor III function as an internalization motif (Amigorena *et al.*, 1992). Draper is always present on the plasma membrane and accumulates at the phagocytic cup during severed neuron engulfment (MacDonald *et al.*, 2006; McPhee *et al.*, 2010). Therefore, the function of Ced-6 could be to remove Draper from the plasma membrane and concentrate it around the phagocytic cup. Along with the NPxY sorting signal, the YxxILI/L-X_{6-12}-YxxL motif of Draper can also function as internalization signal similar to the ITAM

motif of the Fc receptor. Therefore mutagenesis analysis of both sorting signals will provide clues to the mechanism of Draper internalization.

An additional role of Ced-6 in endocytosis could be an involvement with endosome recycling. In the present study, I discovered that Ced-6 is found within a large structure that does not co-localize with clathrin. To study the nature of this complex, Ced-6 expression could be compared with some established endosomal markers. In past studies, the PTB-domain-containing protein ARH has been found on recycling endosomes and co-localized with Rab11. It has also been suggested that ARH is necessary for trafficking of LDLR family members from the endosomal compartment (Nagai *et al.*, 2003). Recent paper suggest that the association between ARH and the β1 subunit of AP-1 is essential for recycling of LDLR from recycling endosomes (Kang and Folsch, 2011).

I have found that Ced-6 can also interact with AP-1-like ARH (Mishra et al., 2002b). In addition, I found that Ced-6 can interact with the α subunit and the β2 subunit of AP-2 with equal affinity. *Drosophila* contains only one β subunit, which is part of both AP-2 and AP-1 complexes. Therefore, Ced-6 might also be involved in the recycling of Yolkless to the oocyte plasma membrane in AP-1-dependent events. Detailed analysis of Yolkless expression in *ced-6* null *Drosophila* should lead to the identification of a potential recycling defect. The *trans* heterozygotes of *yolkless* null allele, which are intermediate in phenotype between wild-type and severely affected *yolkless* allele, can efficiently accumulate the yolk protein, suggesting that in wild-type oocytes Yolkless expression is much higher than required for proper accumulation of yolk proteins (DiMario and Mahowald, 1987). This observation could also indicate that changes in

Yolkless expression or localization due to the recycling defect can be tolerated, as in the case of *ced-6* null *Drosophila*.

It will be of interest to determine the pathway for Yolkless trafficking after endocytosis. In the *Drosophila* oocyte, the polar granules are required for efficient development, and Oskar mutants could be used to study the polar granules (Vanzo et al., 2007)**.** Oskar induces the formation of pole plasm at the posterior pole of oocyte. These pole plasm are constituted of polar granules containing electron-dense ribonucleoproteins. Several studies have demonstrated the role of Oskar in actin organization and endocytosis (Tanaka and Nakamura, 2008). Moreover, Oskar is detected along with yolk protein on endosomes by immuno-EM studies. Oskar is also detected with clathrin at the plasma membrane (Vanzo et al., 2007). A study of co-localization with Ced-6 should provide interesting information about Ced-6 localization and oocyte development.

3.2.2 Regulation of Ced-6

In addition to the AP-2 and clathrin binding sites, the C-terminus of Ced-6 contains information that directs phosphorylation, and two studies have suggested several phosphorylation sites in C-terminus (Bodenmiller *et al.*, 2007; Zhai *et al.*, 2008). One difference between these two studies was the use of *Drosophila* embryo *versus* a *Drosophila* cell line, Kc167. Another was the use or not use of an external stimulus such as the phosphatase inhibitor calyculin-A. However, mass spectroscopy analysis from both studies indicated phosphorylation of Ced-6 at position 338. Amino acid residues at 374, 376, 378, 469 and 480 have been also been found to be phosphorylated upon treatment with external stimuli such as calyculin-A (Bodenmiller *et al.*, 2007). My results show that ectopically expressed Ced-6-GFP displays a step-like profile on SDS-PAGE after

treatment with calyculin-A, suggesting that several residues of Ced-6 are phosphorylated. Mass spectroscopic analysis of *Drosophila* from different developmental stages would likely yield significant clues about the phosphorylation of Ced-6. The prediction of the kinase involved in phosphorylation of Ced-6 C-terminus at specific amino acids using the software GPS 2.0 revealed that several kinases have potential phosphorylation sites in Ced-6 (Table 3.1). This software predicts that CMGCs (including cyclin-dependent kianse (CDKs), mitogen activated protein kinases (MAP kinases) and glycogen synthase kinase (GSK) are potential kinases of Ced-6; these are essential kinases found in all eukaryotes and might have some role during different functions of Ced-6.

Position	Code	Kinase	Peptide
338	S	CMGC	TASTHSA**S**PSSFLPI
338	S	CAMK/MAPKAPK/MAPKAPK	TASTHSA**S**PSSFLPI
338	S	CMGC/CDK/CDC2	TASTHSA**S**PSSFLPI
338	S	CMGC/CDK/CDK4	TASTHSA**S**PSSFLPI
338	S	CMGC/CDK/CDK5	TASTHSA**S**PSSFLPI
338	S	CMGC/MAPK/ERK	TASTHSA**S**PSSFLPI
338	S	CMGC/MAPK/p38	TASTHSA**S**PSSFLPI
338	S	CMGC/CDK/CDC2/CDC2	TASTHSA**S**PSSFLPI
338	S	CMGC/CDK/CDC2/CDK2	TASTHSA**S**PSSFLPI
338	S	CMGC/CDK/CDK4/CDK4	TASTHSA**S**PSSFLPI
338	S	CMGC/CDK/CDK4/CDK6	TASTHSA**S**PSSFLPI
338	S	CMGC/MAPK/ERK/MAPK1	TASTHSA**S**PSSFLPI
338	S	CMGC/MAPK/ERK/MAPK3	TASTHSA**S**PSSFLPI
338	S	CMGC/MAPK/ERK/MAPK7	TASTHSA**S**PSSFLPI
338	S	CMGC/MAPK/JNK/MAPK8	TASTHSA**S**PSSFLPI
338	S	CMGC/MAPK/JNK/MAPK9	TASTHSA**S**PSSFLPI
338	S	CMGC/MAPK/JNK/MAPK10	TASTHSA**S**PSSFLPI
338	S	CMGC/MAPK/p38/MAPK11	TASTHSA**S**PSSFLPI
338	S	CMGC/MAPK/p38/MAPK12	TASTHSA**S**PSSFLPI
338	S	AGC/RSK/RSK/RSK2	TASTHSA**S**PSSFLPI
374	S	Atypical/PDHK	MDELLLN**S**DSDSDFD
374	S	Other/CK2	MDELLLN**S**DSDSDFD
374	S	Other/PEK	MDELLLN**S**DSDSDFD
374	S	STE/STE7/MAP2K7	MDELLLN**S**DSDSDFD
374	S	Other/CK2/CK2a	MDELLLN**S**DSDSDFD
374	S	Other/CK2/CK2b	MDELLLN**S**DSDSDFD
374	S	Other/IKK/IKKb	MDELLLN**S**DSDSDFD
374	S	Other/Other-Unique/KIS	MDELLLN**S**DSDSDFD
376	S	CK1	ELLLNSD**S**DSDFDPR
376	S	CAMK/CAMK1	ELLLNSD**S**DSDFDPR
376	S	CK1/CK1	ELLLNSD**S**DSDFDPR
376	S	Other/CK2	ELLLNSD**S**DSDFDPR
376	S	Other/IKK	ELLLNSD**S**DSDFDPR
376	S	CK1/CK1/CK1a	ELLLNSD**S**DSDFDPR
376	S	CK1/CK1/CK1d	ELLLNSD**S**DSDFDPR
376	S	Other/CK2/CK2a	ELLLNSD**S**DSDFDPR

378	S	Other/CK2	LLNSDSD**S**DFDPRAD
378	S	Other/IKK	LLNSDSD**S**DFDPRAD
378	S	CK1/CK1/CK1d	LLNSDSD**S**DFDPRAD

469	S	CAMK	AAPRRLN**S**VTTGNGL
469	S	STE	AAPRRLN**S**VTTGNGL
469	S	AGC/AKT	AAPRRLN**S**VTTGNGL
469	S	AGC/PKA	AAPRRLN**S**VTTGNGL
469	S	AGC/RSK	AAPRRLN**S**VTTGNGL
469	S	AGC/SGK	AAPRRLN**S**VTTGNGL
469	S	CAMK/CAMK2	AAPRRLN**S**VTTGNGL
469	S	CAMK/CAMKL	AAPRRLN**S**VTTGNGL
469	S	STE/STE20	AAPRRLN**S**VTTGNGL
469	S	CAMK/CAMK1/CAMK1a	AAPRRLN**S**VTTGNGL
469	S	CAMK/CAMK2/CAMK2a	AAPRRLN**S**VTTGNGL
469	S	CAMK/CAMKL/AMPK	AAPRRLN**S**VTTGNGL
469	S	CAMK/CAMKL/CHK1	AAPRRLN**S**VTTGNGL
469	S	CAMK/DAPK/DAPK3	AAPRRLN**S**VTTGNGL
469	S	AGC/PKC/Delta/PKCd	AAPRRLN**S**VTTGNGL

Table 3.1 : Predicted phosphorylation sites in *Drosophila* Ced-6.

Predictions of phosphorylation sites in *Drosophila* Ced-6 and possible kinases were made using GPS 2.0 (http://gps.biocuckoo.org/). Likely sites are serines at positions 338, 374, 376, 378 and 469.

In the *Drosophila* embryo, *Ced-6* mRNA expression was detected by *in situ* hybridization. Ced-6 transcripts are only expressed at embryonic stages 1-3 and stages 13-16 (http://www.fruitfly.org/cgi-bin/ex/bquery.pl?qtype=report&qpage=queryresults&searchfield=symbol&find=ced-6). The levels of protein expression and thus phosphorylation of Ced-6 in embryonic stages 1-3 and 13-16 are not yet clear due to low levels of *ced-6* mRNA produced in these stages.

During the diapausal state there is no yolk protein accumulation. At the onset of vitellogenesis several endocytic proteins are found at the cortical site of endocytosis (present study; Compagnon *et al.*, 2009). Several studies have shown a role of phosphorylation in clathrin-mediated endocytosis (Traub, 2009). To explore of the role of Ced-6 in promoting yolk accumulation, *Drosophila* ovary lysates from the diapausal state and normal state could be analyzed by 2D PAGE. The proteins that change profile under these two conditions could be analyzed with mass spectroscopy. Ced-6 could be identified by immunoblotting. In another experiment the ovary lysate from diapausal *Drosophila* and from normal *Drosophila* could be incubated with GST-Ced-6 and interaction partners could be characterized by mass spectroscopy. *Drosophila* lysates from a variety of different developmental stages and organs such as salivary gland, fat

body and head could be evaluated. This analysis would reveal the Ced-6 interaction partners at different developmental stages.

Five different transcript isoforms of *Drosophila* Ced-6 have been deposited in GenBank, and all these transcripts encode a 517-residue protein. Ced-6 mRNA is expressed in glial cells (Awasaki *et al.*, 2006) and in oocytes (this study). Different splice isoforms of Ced-6 might be expressed in different tissues. The alignment of the 3' UTRs of different isoforms of *ced-6* transcripts show the variation in the sequence (Figure 3.2).

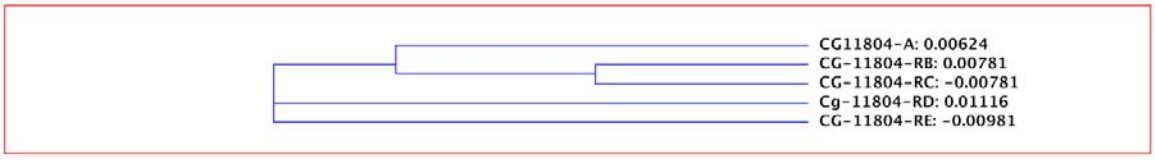


Figure 3.2: Phylogram of *ced-6* transcript isoforms alignment predicts the distance between each using ClustalW.

Recently, Draper was reported to have a role in induction of autophagy. In salivary glands of *Drosophila* larvae, where autophagy is induced, Ced-6 expression was also found to be induced (McPhee *et al.*, 2010). Regulation of Draper expression at the transcriptional level was shown to be induced by ecdysone (Awasaki *et al.*, 2006). The mechanism of transcriptional regulation of Ced-6 is still unknown. The region upstream of the Ced-6 start site might control germline-specific expression *versus* somatic cell expression of Ced-6 transcripts. The upstream regions of all five different transcripts could be cloned into a reporter vector, and lacZ expression could be used to infer locations and levels of protein expression. These types of experiments would give the information on the possible roles of different splice isoforms of Ced-6.

3.2.3 Clathrin-coated pit formation in germ cells

Yolkless-null flies are largely devoid of clathrin-coated pits at the oocyte plasma membrane (Schonbaum *et al.*, 1995). Recent studies suggest that certain levels of cargo, adaptors and accessory factors are necessary for productive clathrin-coated pit formation (Loerke *et al.*, 2009; Mettlen *et al.*, 2010). It seems that Yolkless is the main receptor in *Drosophila* oocytes that is internalized by clathrin-mediated endocytosis. Therefore, study of Yolkless in the formation of the clathrin-coated pit could reveal significant information. The Yolkless receptor truncated to remove the non-canonical dileucine motif or with a mutation to disrupt the FxNPxA sequence could be used to study clathrin-coated pit formation in the *Drosophila* oocyte. Recently it was elegantly shown that with the help of "genomic engineering," a fly gene of interest could be replaced with a modified version of that gene (Huang *et al.*, 2009; Huang *et al.*, 2008). It is known that the strong alleles of *yolkless* are not lethal, therefore a founder knockout of *yolkless Drosophila* line can be maintained. In this *yolkless* knockout line modified *yolkless* can be targeted to its native genomic locus. This set of experiments could reveal whether clathrin-coated pits form *in vivo* in the presence or absence of AP-2. Additionally, such a study might also uncover the long-sought-after and debated sole role of clathrin-mediated endocytosis of yolk protein.

Study of clathrin-coated pit formation could reveal new players in clathrin-mediated endocytosis in oocytes. It is well accepted now that in different tissues clathrin-coated pit formation depends on different proteins. Ovaries from a *Drosophila* germline clone for *AP-2* null phenotype could be studied for yolk protein accumulation. A thin cryosection from these ovaries could be analyzed by electron microscopy for clathrin-coated pit formation. Even though AP-2 is the main constituent of clathrin-coated

vesicles, depletion of > 90% AP-2 does not affect the internalization of certain receptors like LDLR (Keyel et al., 2006a; Motley et al., 2003). However, in these studies AP-2 complex is not completely absent, and analysis of *AP-2* null *Drosophila* germline clones could reveal whether there is an absolute requirement of AP-2 in coated pit formation.

A further approach to studying Yolkless trafficking and yolk protein accumulation could be to study the kinases and phosphatases involved in *Drosophila* Yolkless trafficking. In *Drosophila, skittles (sktl*) encodes a type I phosphatidylinositol-4 phosphate-5-kinase (PIP5K) that is essential for endocytosis (Compagnon *et al.*, 2009). The overexpression of *sktl* impairs the yolk protein endocytosis and results in formation of an abnormal endocytic compartment containing AP-2, Yolkless and Rab5. That study also found that in the absence of Rab5, PtdIns(4,5)P_2 is found in ectopic endosomal compartments in association with AP-2 and aggregates of F-actin (Compagnon *et al.*, 2009). Analysis of this abnormal compartment will might identify proteins involved in Yolkless internalization.

Synaptojanin and its binding proteins endophilin A and Eps15 are not required for clathrin-mediated endocytosis in the *Drosophila* S2 cell line (Korolchuk *et al.*, 2007). The loss-of-function of synaptojanin in the oocyte did not provide the same phenotype as overexpression of *sktl* (Compagnon *et al.*, 2009). It has been suggested that several lipid phosphatases are involved in removal of PtdIns(4,5)P_2 from clathrin-coated pits and early endosomes (Nakatsu *et al.*, 2010). It has also been suggested that phosphatases such as Ship2 and synaptojanin1/2 appear early in clathrin-coated pit maturation, whereas OCRL1 appears at a later stage and could function on endosomes (Nakatsu *et al.*, 2010).

PtdIns(4,5)P_2 levels may be regulated by 34 different *Drosophila* proteins including kinases, phosphatases and phospholipases (Ben El Kadhi *et al.*, 2011). One of

the phosphatases is OCRL, and mutation of this protein causes the oculo-cerebro-renal syndrome of Lowe (Lowe syndrome). Several studies have found OCRL on the plasma membrane, in clathrin-coated vesicles, endosomal vesicles and on the trans-Golgi network (Choudhury *et al.*, 2005; Hyvola *et al.*, 2006; Ungewickell *et al.*, 2004). The knockdown of OCRL causes the PtdIns(4,5)P_2-dependent increase of F-actin in the early endosome-associated pool (Vicinanza *et al.*, 2011). AP-2 was also found in enlarged endosomes that contain PtdIns(4,5)P_2. Knockdown of OCRL1 caused impairment in transferrin recycling by the endosome (Vicinanza *et al.*, 2011). However, knockdown of a PIP5K isoform rescued the defect in transferrin recycling. Thus, both knockdown of OCRL1 and over-expression of Sktl give a similar phenotype. A recent study has identified a *Drosophila* OCRL (dOCRL) that is present in the endosomal compartment containing Rab5, Rab7 and Rab11. Depletion of dOCRL results in formation of a giant abnormal vacuole positive for Rab5, Rab7 and Rab11 (Ben El Kadhi *et al.*, 2011). Studies done in the *Drosophila* oocyte suggest that loss of Rab5 activity leads to the formation of early endocytic intermediate aggregates that contain AP-2 and PH (PLCδ)-GFP, a marker for PtdIns(4,5)P_2 (Compagnon *et al.*, 2009). Loss of Rab5 function also alters the actin organization; actin is found around early endocytic intermediates (Compagnon *et al.*, 2009). OCRL1 proteins from *Drosophila, C. elegans and Dictyostelium discoideum* lack the clathrin- and AP-2-binding motifs present in the vertebrate form of OCRL1 (Choudhury *et al.*, 2009). Rab5 binding is necessary for OCRL1 phosphatase activity (Hyvola *et al.*, 2006).

Considering all these results, it is possible that in the mammalian system a large collection of different phosphatases is necessary for clathrin-coat pit maturation, but in *Drosophila* only few phosphatases, such as OCRL1, are required to remove

PtdIns(4,5)P_2. Therefore, OCRL1 function could be studied in Yolkless endocytosis. Using a transgenic OCRL germ cell line of *Drosophila*, the endosomal structure could be detected by studying oocytes expressing Rab5 and PH (PLCδ)-GFP. Expression of OCRL could also reveal its presence in the oocyte membrane. This same experiment may also show whether dOCRL has phosphatase activity only in the presence of Rab5. In the absence of dOCRL1, AP-2 and Ced-6 should be found in the early endocytic aggregates. These types of experiments should further our understanding of the roles of different adaptors such as AP-2 and Ced-6 in Yolkless trafficking and at the same time clarify the exact role of lipid phosphatase in endosome function.

At the end of *Drosophila* oogenesis, follicle cells display the notable feature of autophagy. During the stage 14 egg-chamber development, follicle cells can exhibit the characteristic autophagy vacuole. These vacuoles are packed with cytoplasmic materials and various organelles (Nezis *et al.*, 2006a). These follicle cells undergo apoptosis and are then phagocytosed by epithelial cells of the oviduct (Nezis *et al.*, 2006a; Nezis *et al.*, 2006b). A recent paper showed that Draper is present in salivary glands and depletion of Draper by RNAi in salivary glands prevented phagocytosis of these cells by hemocytes (McPhee *et al.*, 2010). That study also found that Draper expression is required for autophagy induction in dying salivary glands but not in the fat bodies where autophagy is induced after starvation. One of my experiments suggests the presence of Draper in follicle cells. There is high possibility that Draper also functions in autophagy of follicle cells at stage 14 of ovary development, and then these autophagy-induced follicle cells are engulfed by epithelial cells of the oviduct. Further dissection of the detailed mechanism of Draper interaction and regulation would be very useful for understanding

the importance of Ced-6 during autophagy, phagocytosis and clathrin-mediated endocytosis.

3.3 Concluding comments

In this dissertation, I presented data that demonstrate the roles of two adaptor proteins in the internalization of Yolkless by clathrin-mediated endocytosis. The recognition of Ced-6 as a member of the CLASP family provides a new perspective on the function of clathrin-mediated endocytosis. This finding raises a question regarding the role of clathrin-mediated endocytosis in phagocytosis. Yolkless endocytosis is critical for the survival of *Drosophila*, but manipulation of the endocytic pathway of Yolkless will reveal details of a fundamental process conserved across species.

4.0 MATERIALS and METHODS

4.1 *Drosophila* stocks

Drosophila (Canton-S) was used for all experiments as a wild-type fly. GFP-Clathin light chain (GFP-CLC) expressing *Drosophila* were generated (BestGene Inc., Chino Hills CA) using clathrin light chain plasmid. GFP-CLC, a kind gift from Henry C. Chang (Chang *et al.*, 2002), was cloned in pUASP vector between *Not*I and *Xba*I restriction endonucleases. Triple Gal4 driver (Lynn Cooley lab, Yale University) was used to cross with Clathrin-LC-GFP expressing *Drosophila* for germ cell expression of clathrin light chain. The null allele for Ced-6 was a kind gift by Takeshi Awasaki (*w; ced-6j26/cyO,act-GFP*). The *Yolkless* strain was received from Bloomington Stock Center.

4.2 Diapausal condition

Diapausal condition was obtained by shifting the wild-type *Drosophila* (Canton-S) to 11˚C and a 14 h/10 h dark-light cycle for 72 h. For the experiment, these flies were kept for another 36 h at the same temperature or released into room temperature and normal dark-light cycle. *Drosophila* ovaries were dissected in Schnieder cell media (Gibco) and stored on ice in PBS. Whole ovaries were resuspended in SDS sample buffer to analyze on SDS-PAGE. To visualize the trypan blue (0.04% in PBS) or thrombin-cleaved mcRFP-RAP endocytic tracer, dissected ovaries incubated in mcRFP-RAP (0.16 mg/ml in PBS) were kept at 25˚C water bath for 20 min. Ovaries were washed in PBS and fixed in 4% PFA (Electron Microscopy Sciences) for 30 min at room temperature. Images were captured on Olympus FV1000. In some experiments fixed ovaries were blocked in 2% BSA and 0.3% Triton X-100. Blocked ovaries were incubated with AlexaFluor488-

Phalloidin to visualize actin.

4.3 Plasmids

The plasmid encoding Ced-6 (1-517) was PCR amplified from LD19266 (DGRC, Bloomington Indiana), and the amplicon was ligated directionally between *EcoR*I and *Xho*I in pGEX4T-1. To construct GFP-Ced-6, the Ced-6 sequence was ligated to the N-terminus of green fluorescent protein in pEGFP-N2 by inserting an amplified PCR template from the same clone between *Xho*I and *Kpn*I sites. Different regions of Ced-6 were produced by either PCR amplification or by introducing stop codons or amino acid reversals by QuikChange mutagenesis (Stratagene/Agilent technologies) in the GST-Ced-6 plasmid (amino acids coding region 1-300, 285-517, 370-517, 401-517, 430-517, 401-468, 401-500 or DPF-APA). His_6 -Ced-6 (285-517) was cloned between *EcoR*I and *Xho*I restriction site in pET28 vector. GST-AP-2 α_c appendage and GST-AP-2 β2 appendage were described previously (Traub *et al.* 1999, Edeling *et al.*, 2006; Mishra *et al.,* 2005) and GST β2 Y888M was created by QuickChange mutagenesis in GST-β2**.**

Drosophila receptor associated protein (RAP) was created by PCR amplification of clone LD29322 (DGRC Bloomington). The fusion protein was generated by directional ligation of the PCR amplified open reading template encoding the region 31-380 amino acids lacking the N-terminus signal sequence between *EcoR*I and *Xho*I in pGEX4T1 to create a tendem GST-mcRFP-RAP fusion protein.

Tac (CD25; the IL-2 receptor α -subunit) in pcDNA3.1 was a kind gift from Rebecca Hughey (University of Pittsburgh, Pittsburgh, PA, USA). The Yolkless cytosolic tail (aa 1829-1937) was PCR amplified from clone RE59172 (DGRC Bloomington, Indiana) and ligated between *EcoR*V and *Not*I restriction endonuclease

sites in Tac-pcDNA3.1. The same amplicon was inserted in the pet28 vector to express Yolkless as a His_6 fusion protein. Mutagenic replacement of amino acids (LL1919AA; and A1842Y) or additions of stop codon in Yolkless were generated by QuikChange mutagenesis.

GULP1 was PCR amplified from the GenBank clone BC068525 and directionally ligated between *EcoR*I and *Xho*I in pGEX4T1 and as a tandem dimer in Tomato-RFP (tdRFP). Plasmids encoding the GST-ARHC1 (Mishra *et al.*, 2005), the GST-AP-2-α_c (Traub et al., 1999a) and AP-2-β2 appendage (Edeling *et al.*, 2006a; Thieman *et al.*, 2009) and associated mutatnts have been described. Gulp-GFP was kindly provided by Kodi S. Ravichandran (University of Virginia, Charlottesville, VA). All the positive clones were validated by automated dideoxynucleotide sequencing (Genewiz Inc. NJ) by using appropriate primers. All the oligonucleotides used are listed in Appendix Table A.1.

4.4 Antibodies

The monoclonal antibodies (mAbs) anti-clathrin heavy chain TD.1 (Nathke *et al.*, 1992) and the anti-AP-1/2 β1/β2-subunit mAb 100/1 (Ahle *et al.*, 1988) and affinity-purified GD/1 antibodies (Traub *et al.*, 1995), affinity-purified rabbit anti-epsin 1 polyclonal (Drake *et al.*, 2000) were used. The following antibodies were generous gifts: anti-Tac antisera was provided by Juan Bonifacino (NIH, Bethesda, MD, USA), affinity-purified rat anti-Yolkless was a kind gift by Anthony Mahowald (Schonbaum *et al.*, 2000), rat anti-Ced-6 serum was provided by T. Kawasaki (Awasaki *et al.*, 2006), rat anti-liquid facets was provided by B. Zhang, the affinity-purified rabbit anti-GFP kindly provided by Phyllis Hanson (Dalal *et al.*, 2004), the AP-2 α-subunit mAb AP.6 (Chin *et al.*, 1989)

was kindly provided by Frances Brodsky and the rabbit anti-*Drosophila* yolk protein 1-3 (YP1-3) was kindly provided by Mary Bownes (Bownes *et al.* 2002).

The following commercial antibodies were used: an anti-AP-2 α subunit mAb clone 8 (BD Transduction Laboratories), affinity-purified anti-tubulin mAb E7 (Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit anti-eps15 and anti-actin mAb C4 (Santa Cruz Biotechnology) and anti-Tac mAb 7G7B6 (Ancell Immunology Research Products, Bayport, MN, USA). Anti-Tac mAb 7G7B6 was conjugated with Alexa fluorophore 546 antibody labeling kit as per the instructions provided by the manufacturer (Invitrogen). Goat anti-mouse or anti-rabbit secondary antibodies conjugated to either Alexa Fluor4 88 or Alexa Fluor 568 were purchased from Invitrogen, whereas goat anti-mouse, anti-rat or anti-rabbit antibodies conjugated to Cy3 or Cy5 were from Jackson Labs. HRP-conjugated donkey anti-mouse and anti-rabbit secondary antibodies used for immunoblots were purchased from GE-Healthcare. The alkaline phosphatase-conjugated sheep anti-DIG (Roche Applied Science) was used in *in situ* staining.

4.5 SDS-PAGE

Polyacrylamide gel was used to resolve the protein samples. Polyacrylamide gels were prepared with a modified ratio of acrylamide to bis-acrylamide (30:0.4) stock solution. After resolving the samples on polyacrylamide gels, Coomassie blue dye was used to visualize the proteins. For immunoblots, SDS-PAGE resolved samples were transferred onto nitrocellulose in ice-cold transfer buffer (15.6 mM Tris and 120 mM glycine). Transferred nitrocellulose membranes were blocked for 2 hrs or overnight in 5% fat free milk in 10 mM Tris–HCl (pH 7.8), 150 mM NaCl and 0.1% Tween 20 (TBST). Different

pieces of blocked nitrocellulose membrane were incubated with appropriate primary antibodies as mentioned in the respective figure legends. Horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-rat immunoglobulin G were used to visualize with enhanced chemiluminescence (Danville).

4.6 Reagents and protein purification

Liposomes were prepared by mixing appropriate amount (weight/volume) of cholesterol (10%), phosphatidyl-ethanolamine (35%), phosphtidylcholine (35%) and 10% phosphatidylinositol(4,5)-bisphosphate (PtdIns[4,5]P_2) or 0.2mg/ml Folch brain extract. As a negative control, cholesterol and PtdIns was replaced with 30% phosphatidylserine. After the incubation with liposomes samples were sedimented at 20000 *g* at 4°C. Appropriate amount of supernatant and pellet were resolved on SDS-PAGE and stained with Coomassie Brilliant Blue to visualize the binding of proteins.

Rat brain cytosol was purified by homogenizing frozen rat brains (PelFreez) at 4°C in 25 mM HEPES-KOH, pH 7.2, 250 mM sucrose, 2 mM EDTA and 2 mM EGTA supplemented with 1 mM PMSF and complete protease inhibitor cocktail (Roche Applied science). Homogenized rat brains were then differentially centrifuged to collect the supernatant (Traub *et al.*, 1996; Traub *et al.*, 1993). Protein concentration was determined by Bradford assay (BioRad). Cytosol was frozen at -80°C for further use.

Plasmids encoding GST or the different GST-fusion proteins were transformed into *E. coli* BL21or *E. coli* BL21-codonPlus, whereas His_6- fusion plasmids were transformed into *E. coli* BL21 (DE3). An overnight 10-ml bacterial culture was inoculated into 500 ml sterile LB media in an Erlenmeyer flask with an appropriate antibiotics and left to grow at 37°C for 2-3 hours with shaking. At mid-log phase bacterial cultures were induced

with 100 μM or 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at room temperature for GST or His_6-fusion proteins induction, respectively, and induced bacterial cells were harvested after incubation for 3 hours and frozen at -80°C for further use. Frozen bacterial pellets were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM sodium chloride, 0.2% (w/v) Triton X-100, 10 mM β-mercaptoethanol) in the presence of 1 mM PMSF and complete protease inhibitor (Roche Applied Science) on ice and contaminating genomic DNA was sheared by sonication. Lysates were further centrifuged to remove insoluble debris. The supernatants containing GST or His_6-fusion proteins were collected on glutathione-Sepharose or Nickel-NTA-agarose beads, respectively. Beads were washed, and GST or His fusion proteins were eluted with 10 mM glutathione or 100 mM imidazole, respectively. Eluted proteins were dialyzed against PBS containing 1 mM DTT. Thrombin was used to cleave GST-fused proteins from GST-Aepharose. To stop the thrombin action, 25 μM final concentration of D-Phe-Pro-Arg chloromethyl ketone (Calbiochem), an irreversible thrombin inhibitor, was added. Purified AP-2 core complex was kind gift from David Owen (MRC, Cambridge, U.K.).

4.7 Protein-binding assays

GST-based pull-down assays, electrophoresis and immunoblotting were performed as described (Mishra et al., 2002a; Mishra et al. 2002b; Jha et al., 2004; Thieman et al al., 2009). Approximately 5-200 μg of appropriate proteins were immobilized on either packed 10 μl Ni-NTA-agarose or packed 25μl GST-sepharose by mixing at 4°C for 1-2 hours. Unbound proteins were removed by washing twice in assay buffer (25 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 5 mM magnesium acetate, 2 mM EDTA, 2

mM EGTA, 1 mM DTT). Immobilized proteins on beads were used in pull down assays. Rat brain cytosol equilibrated in assay buffer in presence of 1 mM PMSF was centrifuged at 100000 *g* for 20 min. at 4°C to remove any traces of aggregated proteins. Rat brain cytosol (~8 mg/ml) in final volume of 250 µl equilibrated in assay buffer was incubated at 4°C for 1 hour with immobilized GST beads. After the pull down assay, the beads were washed four times in ice cold PBS and resuspended in SDS sample buffer in a final volume of 80 µl. Between 10 and 12.5% of the packed beads were resolved on SDS-PAGE. Gels were either stained with Coomassie Brilliant Blue or transferred onto nitrocellulose membrane for immunoblot analysis. Direct protein-protein interactions using the purified proteins were performed in PBS in presence of 0.1 mg/ml BSA.

For liposome-binding assays, a final reaction of 200 µl contained control or PtdIns(4,5)P_2 liposomes (0.4 mg/ml), BSA (100 µg/ml) and 5, 10 or 20 µg of thrombin cleaved Ced-6 (1-300) in assay buffer (Mishra *et al.*, 2002b). The binding experiments were performed at room temperature for 30 min. Liposomes was sedimented after centrifugation at 20,000 *g* for 15 min at 4°C. Appropriate amount of supernatant and pellet were mixed with SDS-sample buffer to resolve on SDS-PAGE and visualized by Coomassie Brilliant Blue.

4.8 Whole-mount *in situ* hybridization

Whole mount *in situ* antisense riboprobe was generated from the C-terminal of *Drosophila* Ced-6 by polymerase chain reaction using set of oligoes with *Dm* Ced-6 forward 5'CGACACCAATTCCACCACAG3' and *Dm* Ced-6 reverse, 5'-**GCGTAATACGACTCACTATAGGGA**GGATCCAAACTCTCCAGCG-3'. T7 polymerase promoter sequence was added to the reverse primer (bold letters) to generate

the riboprobes. Antisense riboprobes generated for *Drosophila*, clathin heavy chain, AP-2 α, Oskar, Dab, receptor-associated protein (RAP), Yolkless, skittle, Numb are given in Appendix Table A.1. PCR-amplified template was purified using QIAquick PCR cleaning kit (Qiagen), and purified template (~500 ng) was used to generate digoxigenin-labeled riboprobes as per the manufactures instruction using digoxigenin-11 UTP RNA labeling mix and 40 units of T7 RNA polymerase (Roche Applied Science). The RNA probes were fragmented at 65°C for 10 minutes in 120 mM sodium carbonate, 80 mM sodium bicarbonate (pH 10.2) for better penetration in fixed tissues. Labeled and fragmented riboprobe was purified using 0.2 M sodium acetate (pH 3.5), 4 M lithium chloride and ethanol at -20°C overnight and washed in 70% ethanol in DEPC-treated water at 4°C. Precipitated riboprobes were resuspended in hybridization buffer containing 50% formamide, 5x SSC, 100 μg/ml sonicated salmon sperm DNA, 50 μg/ml heparin and 0.1% Tween 20 in DEPC-treated water. The concentration of riboprobes was determined by dot blot using serial dilutions of riboprobes on nylon membrane.

Aedes female ovaries were dissected previtellogenic and 6 hour or 20 hour after the blood meal. Ovaries were fixed in 4% PFA-DMSO (5:1) at room temperature for 30 min. Ovaries were washed in PBST (PBS containing 0.1% Tween-20 and were subjected to proteinase K treatment (8 μg/ml in PBST) for 10 min at room temperature. Ovaries were again washed in PBST and refixed in 4% PFA/DMSO (5:1). Fixed ovaries were prehybridized in hybridization buffer for 60 min at 55°C. Hybridization buffer was replaced with fresh hybridization buffer supplemented with or without digoxigenin-labeled riboprobes and incubated overnight in water bath with shaking. After hybridization ovaries were washed in hybridization buffer with few changes of buffer for 5 hours at 55°C. The hybridized ovaries were blocked in 1% normal goat serum in PBST

for 10 minutes followed by incubation for 60 min with an alkaline phosphatase-conjugated anti-digoxigenin mAb (Roche Applied Science) containing 1% goat serum in PBST. After extensive washing, ovaries were stained by using 5-bromo-4-chloro-3′-indolyphosphate p-toluidine (BCIP) and nitro-blue tetrazolium (NBT).

Appropriate *Drosophila* wild-type (CS WT) ovaries were fixed in 4% para-formaldehyde at room temperature for 30 minutes, washed in 1X PBS and stored in 100% methanol at -20°C. To initiate the hybridization, methanol was replaced with 1 ml of cold acetone (prechilled at -20°C). After incubated at -20°C for 8 min, acetone was replaced with cold methanol. Methanol was replaced with PBTw by exchanging in serial dilution of methanol in PBTw. Finally *Drosophila* ovaries were washed twice in PBTw (PBS, 0.2% BSA and 0.1% Tween-20). Ovaries were then treated with 10 μg/ml Proteinase-K in PBTw for 5 min at room temperature. Ovaries were prehybridized in hybridization buffer without the antisense riboprobe (50% formamide, 5X SSC, 5 mM EDTA, 0.1% Tween-20, 0.1 % CHAPS in presence of 50 μg/ml heparin and 1 mg/ml total RNA) at 65°C for 1 h in a hybridization oven. Hybridization buffer (1 ml) along with 1 μl DIG-labeled antisense riboprobe was heated at 80°C for 2 min and quenched on ice. Hybridization buffer was replaced with fresh buffer along with riboprobes and incubated at 65°C overnight with mild shaking. Ovaries were washed twice in 50% formamide, 2X SSC, 0.3% CHAPS at 65°C for 30 minutes followed by two washes for 15 min in 2X SSC and 0.3% CHAPS at 65°C. The final four washes were in 0.2X SSC and 0.3% CHAPS at 65°C at room temperature. Washing buffer was replaced by MAB buffer (0.1 M maleic acid, pH 7.5, 150 mM NaCl), and ovaries were incubated for 15 min at room temperature. After 1 hour at room temperature in blocking solution (2% blocking reagent (Roches Applied Science) in 50 ml MAB and 5% lamb serum), blocking solution

was replaced with fresh blocking solution containing anti-DIG alkaline phosphatase antibody (1:2000 dilution). Antibody was pre-adsorbed, and ovaries were incubated for 2 h with mild shaking at room temperature. Ovaries were washed with multiple exchanges with PBTw overnight at 4°C. Similar washing were performed five times at room temperature for 30 minutes each washing. For staining, PBTw was replaced with staining solution (0.1 M Tris-HCl [pH 9.5], 0.1 M NaCl, 50 mM $MgCl_2$, 0.1% Tween-20 and 1.2 mg/ml Levamisol) and incubated for 10 minutes with an exchange of staining solution once. Color reaction was performed using pre-warmed BM-Purple (Roche Applied Science) in the dark until the visualization of color reaction. For microscopy, stained ovaries were preserved in 50% glycerol in PBS. Oriented ovaries were viewed with a Zeiss stereo microscope. Images were processed in Adobe Photoshop-CS3.

4.9 Cell culture and maintenance

HeLa SS6 (Elbashir *et al.*, 2001) and HT-1080 (Rasheed *et al.*, 1974) cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Cultures were maintained by passaging trypsinized cells. Cells were maintained at 37°C and 5% CO_2 in humidified growth chamber. For transfection, the HeLa SS6 cells were transferred to sterile 10-mm round glass coverslips. At about 60% confluency, cells were transfected using Lipofectamine-2000 (Invitrogen) as described earlier (Keyel et al., 2006b; Mishra et al., 2008). Approximately 0.5-1 μg of purified plasmid was used to transfect the cells in wells of a 6-well plate.

4.10 siRNA-mediated knockdown

siRNA-mediated depletion of clathrin heavy chain or AP-2 α subunit in HeLa cells was performed using Oligofectamine (Invitrogen). siRNA sequences are described in Keyle *et al.* (2006). After the first siRNA transfection (24 hours), HeLa cells were trypsinized and replated in 24 well plates either with or without coverslips. HeLa cells were again re-transfected with siRNAs. The tac-Yolkless siRNAs were transfected using Lipofectamine-2000 (Invitrogen) 6 hours after the second transfection of siRNA. Knockdown experiments were done twice and efficiency of knockdown was confirmed by immunoblot using appropriate antibodies.

4.11 Immunofluorescence

Hela cells were transiently transfected with Tac-Yolkless cytosolic tail alone or co-transfected with Ced6-6-GFP overnight to visualize Tac internalization. For experiments involving transferrin uptake, cells were starved for 1 hour in media containing DMEM supplemented with 25 mM HEPES, pH 7.2 and 0.5% BSA to displace the bound transferrin. To track the receptors and Tac-chimeras internalization, cells on coverslips were incubated with the anti-Tac mAb clone 7G7B6 along with 20 μg/ml transferrin conjugated to AlexaFluor568 (Tfn568; Invitrogen) or 20 μg/ml transferrin conjugated to AlexaFluor633 (Tfn633; Invitrogen) in starvation medium for 1 h on ice at 4°C and chased at 37°C for 2-20 minutes. In some experiments Tac antibody conjugated with Alexa546 was used to visualize internalized population. Internalization was stopped by washing the cells in cold PBS followed by fixation in 4% paraformaldehyde. Control cells were fixed without warming at 37°C, and cells were permeabilized in 0.3% saponin and 10% normal goat serum in PBS before immunostaining.

For whole mount immunofluoresence, *Drosophila* ovaries were dissected and fixed in 4% paraformaldehyde for 30 min at room temperature. Fixed ovaries were blocked in blocking buffer containing 2% BSA, 0.3% Triton X-100 and 5% normal goat serum in PBS and incubated with primary antibody overnight at 4°C in blocking media. Ovaries were washed for five times with an interval of 20 min in between and incubated with fluorophore conjugated secondry antibody at room temperature for 1 h. Where indicated, ovaries were incubated with Alexa488-phalloidin (1:500 dilution; Invitrogen) for 60 minutes in the dark to counterstain actin and/or Hoechst 33258 (2 μg/ml in PBS) to counterstain nuclei. After several washing, ovaries were mounted in Gelvatol mounting media.

For calyculin treatment, Hela cells transfected with Ced-6-GFP in 6-well plates were incubated with or without 100 nM calyculin A (LC Laboratories) in a water bath maintained at 37°C for 60 min. Cells were washed in PBS containing 100 nM calyculin A. Cells were detached gently by scrapping from the surface and centrifuged at 1000 *g* for 3 minutes at 4°C. Cells were immediately solubilized in SDS sample buffer. Solubilized cells were sonicated to shear the genomic DNA and the samples were resolved on SDS-PAGE and transferred on nitrocellulose for immunobloting.

4.12 Microscopy

All confocal imaging was performed with an inverted Olympus Fluoview 1000 (FV1000) confocal microscope (Melville, NY) using sequential line scanning. An argon laser with 488-nm excitation and two helium-neon lasers with 543- and 633-nm excitation were used to acquire the images through a PlanApomat 60 X 1.40 NA objective with a 488/543/633 dichroic. The emitted light signal was separated with SDM560 and SDM

640 beam splitters and passed through either bandpass filter BA505-525 (green channel) or BA560-600 (red) and long-pass filter BA660IF (Cy5) or diffraction grating that pass 500-530-nm (green) or 552-625-nm (red) light or a BA65oIF filter (Cy5) before collecting the image. All the acquired digital TIFF images were processed in Photoshop-CS4 (Adobe).

4.13 EM analysis

For electron microscopy *Drosophila* ovaries were fixed in 2.5% glutaraldehyde in PBS for 10 minutes at room temperature. Ovaries were washed three times in PBS for 15 minutes. Samples were stained with 1% osmium tetroxide containing 1% potassium ferricyanide for 1 h. Samples were dehydrated with ethanol and embedded in epon resin. Thin sections of ovaries were cut using an ultramicrotome, stained with uranyl acetate and lead citrate. Sections were then examined by transmission electron microscope (JEOL 1210) and images collected with an ATM digital camera.

4.14 Software and websites used

For data analysis, ImageJ (Abramoff et al., 2004) was used to quantify the western blot band intensity. Photoshop CS4 and Illustrator CS4 (Adobe) were used for image processing. Microsoft Office was used for word and data processing. Endnote X5 was used for bibliography. GPS1 was used for the identification of kinase analysis for predicting the phosphorylation site (Xue et al., 2008). Following websites were used for data mining:

http://www.ncbi.nlm.nih.gov/pubmed/

http://www.ncbi.nlm.nih.gov/gene/

https://dgrc.cgb.indiana.edu/

http://flystocks.bio.indiana.edu/

http://www.uniprot.org/help/uniprotkb

http://prodata.swmed.edu/promals3d

http://swissmodel.expasy.org/

APPENDIX

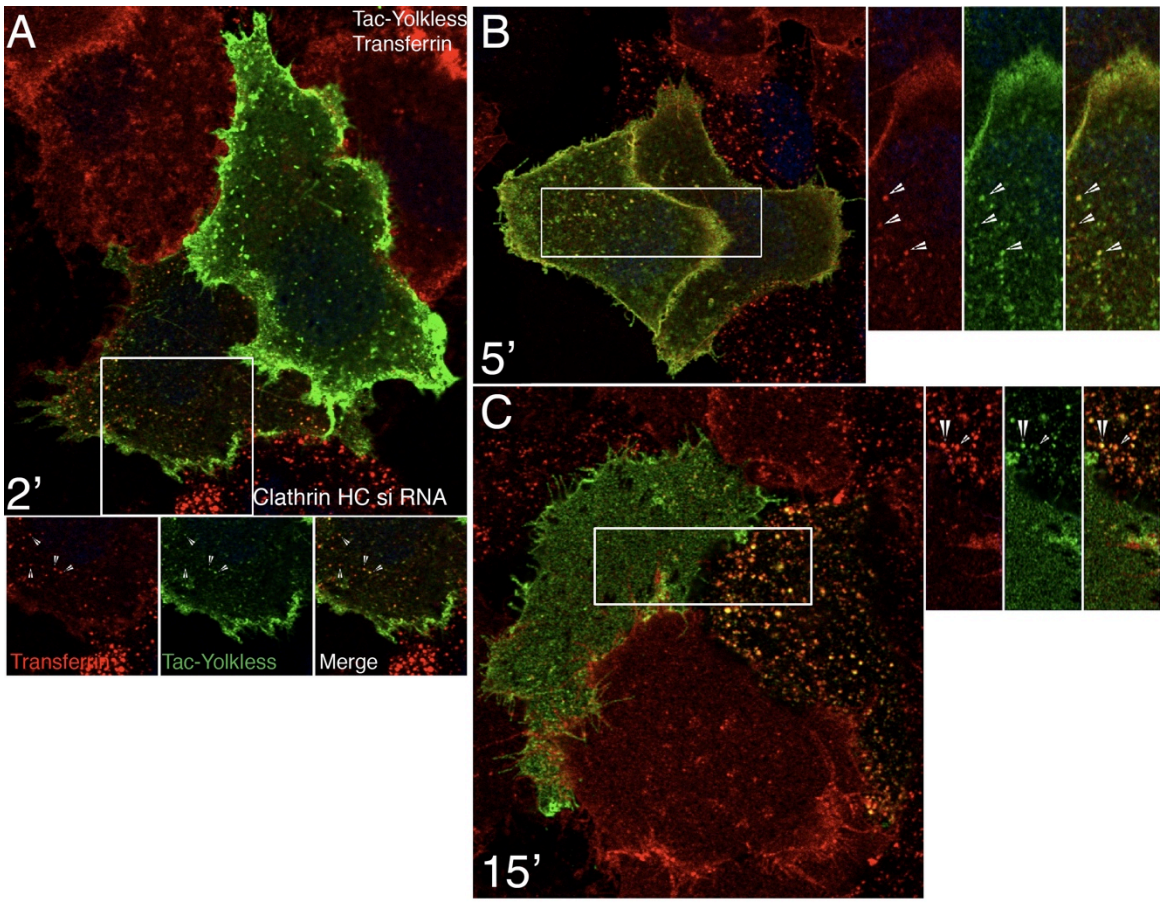


Figure A.1: Inhibition of Tac-Yolkless internalization by depletion of clathrin.

Ectopic expression of Tac-Yolkless in Clathrin HC knockdown in HeLa cells were starved for 1 h in starvation media and pulsed on ice for 1 h using mouse anti-Tac antibody and 20 μg/ml transferrin-568 and chased at 37°C for 2 min (A), 5 min (B) or 15 min (C). Cells were fixed and permeabilized and probed with donkey anti-mouse-Alexa488 to visualize Tac. Representative boxed area represents the partial or complete loss of internalization of Tac-Yolkless or transferrin. Representative single optical sections are shown.

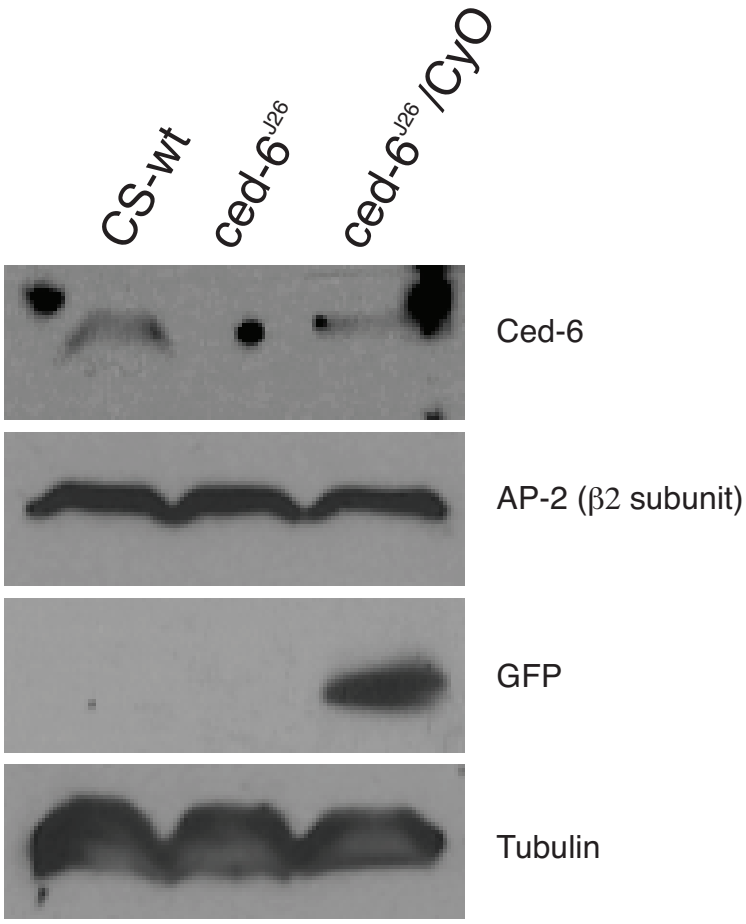


Figure A.2: Western blot confirming the loss of Ced-6 in heterozygote

Immunoblot of total ovary lysate from Canton S wild-type (left lane), Ced-6^{J26} (middle lane) and Ced-6^{J26}/Cyo (last lane). Equal amounts of protein were resolved on SDS-PAGE and immunoblotted for rat anti-Ced-6, MAb 100/1 anti-AP-2 β, affinity purified rabbit anti-GFP or MAb anti-tubulin.

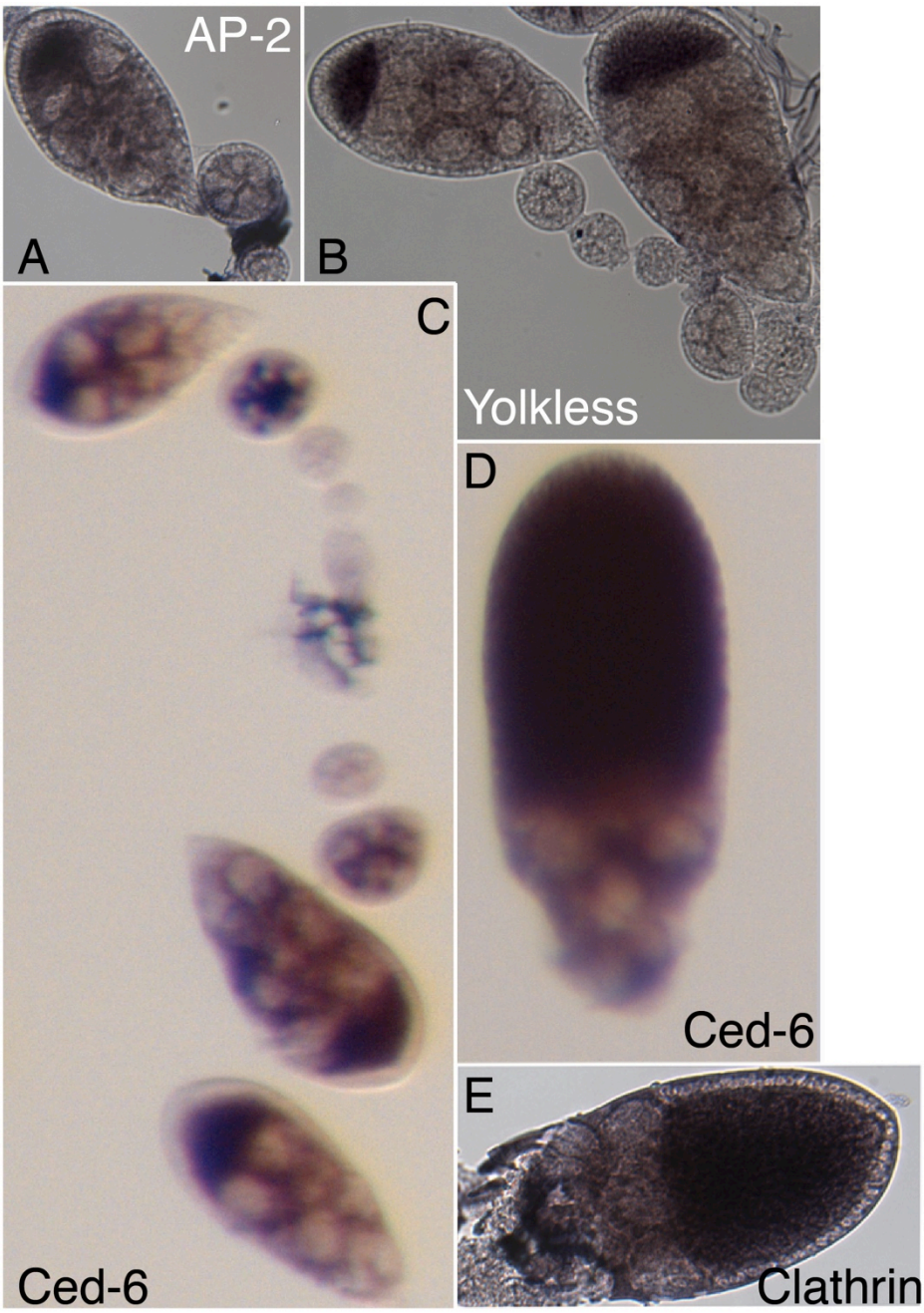


Figure A.3: Different endocytic transcripts in *Drosophila* oocyte.

Whole-mount *in situ* hybridization analysis showing the position of indicated transcripts in stage 10 oocytes in purple: (A) α subunit of AP-2; (B) Yolkless; (C and D) Ced-6 and (E) Clathrin heavy chain.

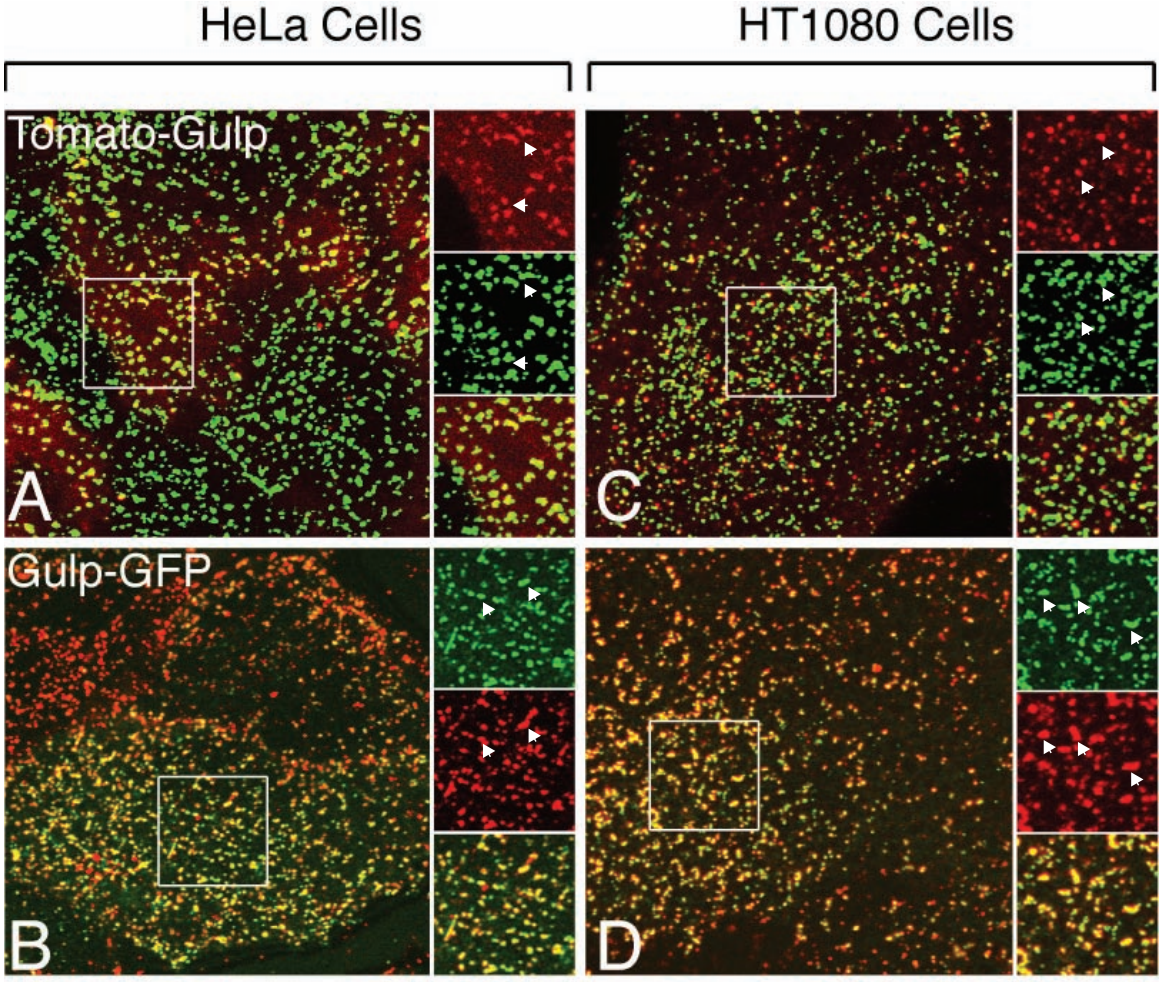


Figure A.4: PTB domain containing protein Gulp1, a human homolog of Ced-6, shares a similar compartment in different cell types.

Transient expression of Tomato-Gulp in epithelial HeLa cells (A) or fibroblast HT1080 cells (C) or Gulp-GFP in epithelial HeLa cells (B) or fibroblast HT1080 cells (D) confirms the steady state distribution along with AP-2 either decorated in green (panels A and C) or in red in (panels B and D).

Table A.1: Oligonucleotide sequences

Name	Oligonucleotide sequence
Aae Trephin F	GCAGCGCACGATCACGTGTTCGC
Aae Trephin R	GCGTAATACGACTCACTATAGGGAGAAGTCCTGCTCCATCCATTGGC
Aae Vitellogenin F	CGCTGCAAGCACGACAAAAGC
Aae VitellogeninR R	GCGTAATACGACTCACTATAGGGAGAGACGCACGTGCTATTGTCCGC
Dm-Yolkless-CT 5'	TTTGATATCACGGATCTCAACATTAACATGC
Dm-Yolkless-CT-3'	CTCGCGGCCGCTTACGACACAAATCGTGCATTC
DM-Yolkless-ct-FxN to AxA	CTCAACATTAACATGCATGCCCAGGCTCCGCTGGCGACGCTCG
GC - DM-Yolkless-ct-FxN to AxA	CGAGCGTCGCCAGCGGAGCCTGGGCATGCATGTTAATGTTGAG
Tac-Yolkless-1885 stop	CAGCGCCACGTCCTAGTTCGCGGCTC
GC - Tac-Yolkless-1885 stop	GAGCCGCGAACTAGGACGTGGCGCTG
Tac-Yolkless 1906 stop	GTCCACGACAGTAGGCGTCCGGCGATC
GC - Tac-Yolkless 1906 stop	GATCGCCGGACGCCTACTGTCGTGGAC
Tac-Yolkless 1919 stop	CAGGAATTGCTTCTCTAGAGCCCCAGCAGAGTG
GC - Tac Yolkless 1919 stop	CACTCTGCTGGGGCTCTAGAGAAGCAATTCCTG
Dm-Yolkless F	CGTCTGGTGGCCGAAGGCGAACGGTGT
Dm-Yolkless R	GCGTAATACGACTCACTATAGGGAGATCACATATCTTCCACGGCC

Dm Boca- F	GCAAACACGTCTTGTTTTGC
Dm Boca- R	GCGTAATACGACTCACTATAGGGAGAGTTCTCGATGGTTACGCCCTTGC
Dm Oskar- F	ATGGCCGCAGTCACAAGTGAATTC
Dm OSkar- R	GCGTAATACGACTCACTATAGGGAGACTTGATGCTCGATATCGTGATTCC
Dm RAP- F	CTTAAGCCCTACAAGAAATTCCAC
DM RAP- R	GCGTAATACGACTCACTATAGGGAGAGGTTTTAATGGACTCCAGTTCTTTAAC
Dm Megalin F	GATGAGGAAACGGAGTTCACTTG
Dm Megalin R	GCGTAATACGACTCACTATAGGGAGACACGTTGCAATGGGCAGGTTC
Yolkless 5' add EcoRI	AAAGAATTCCGGCAGCGCGGCCACACG
Yolkless 3' BamHI	AAAGGATCCGTTTGCTTTCCTGTTGAGCAGAC
Dm CHC F	CTCGTGGTCAAGGGACAATTC
DM CHC R T7	GCGTAATACGACTCACTATAGGGAGACAAAGCGGTCTCTACCACCTG
Dm alpha F	GACGTTTTGGCCACCGTCCTG
Dm alpha R	GCGTAATACGACTCACTATAGGGAGACACGTTCAGCTTAAGCGCATCC
Dm Disabled F	TGCTGGATAAATCTATATATACAATC
Dm Disabled R	GCGTAATACGACTCACTATAGGGAGACCACATCTACAAGTCCCTGGC
GFP-CLC-F	TAAAGCGGCCGCCACCATGGTGAGCAAGGGCG
GFP-CLC-R	GCTCTAGATTAGGCGAGTGCGTAATTAAAAC
Dm Yolkless (1-2914) F1	GGGGTACCATGTGCCAGGCCGAGCAC
DM Yolkless (1-2914) R1	CGGAATTCCATTGGGCGTG
Dm Yolkless (2914-4700)F2	GCGAATTCCGCTGGCCGCCAG
Dm Yolkless (2914-4700) R2	TAAAGCGGCCGCACATGCGTC
Dm Yolkless (4700-5814) F3	TAAAGCGGCCGCATTGTGCACG
Dm Yolkless (4700-5814) R3	GCTCTAGATCACATATCTTCCACGGCCATC

pUASP-F-Seq.oligo	GTGCGTTAGGTCCTGTTCA
pUASP-R-Seq. oligo	GTTTACTCTTGACCATGGG
DmLpR2-F	AAGAGGGACTGTGCGGATGGC
Dm LpR2-T7	GCGTAATACGACTCACTATAGGGAGACACAATTGACGTCATTTCCGGTC
Dm Pacsin - insitu 5'	GACCTGGAGGATCTGTTCAAG
Dm-Pacsin 3' in situ	GCGTAATACGACTCACTATAGGGAGACTGGTCATTGTTGATCGTGTGGG
Dm syndapin QC EcorI	GCGACAACGTGCTGGTGGAATTCGGTGAGCCAGGCGTG
Hs Pacsin QC EcorI	CGAATCCATTCGACGACGAATTCACCTCGGGGACGGAAGTG
GC - Dm syndapin QC EcorI	CACGCCTGGCTCACCGAATTCCACCAGCACGTTGTCGC
GC - Hs Pacsin QC EcorI	CACTTCCGTCCCCGAGGTGAATTCGTCGTCGAATGGATTCG
Dm Yolk protein 1 F	ACGCGTCGACCACCTGTCGCAGATCCACC
Dm Yolk protein1 Rev	ATAAGAATGCGGCCGCTTACTGGTAGTCCTTGCTGTC
DM Numb	CGAAAGCAGCGACGCGACAAGG
Dm Numb Rev	GCGTAATACGACTCACTATAGGGAGACTGACACAGTTGGCTCACAGAATC
Dm NAK-Forward	CCACCCGGACTGCGCGAGAAG
DM NAK Rev	GCGTAATACGACTCACTATAGGGAGAATCGCGAAGTTTCTTCACAATTGG
Aae-Trephin-SacII	TCCCCGCGGATGGCGTTCTTCAAGAGCATC
Aae-Trephin with NotI 3'	GACGCGGCCGCTCAGTTAGAGCAAATTGCAG
RAP-5' with Kozak	GCGAATTCGCCACCATGAAGTACAGCAAGGAGGCCAAC
RAP-Rev-seq	CAACTTGGCCATGCGAAATGGC
RAP with XbaI	GCTCTAGAATGGTTAGGTCAGCTCTCGTTGTTG
RAP with NheI	CGGCTAGCAATCAGATTGTAGGTGTCCAAGTC

PWIZ(-) Seq Oligo	CGAATAGAAACTCAC
pWIZ (+) SeqOligo	CCAATTTGAAACTCAG
RAP with NOtI for pWIz	TTTGCGGCCGCATGGTTAGGTCAGCTCTCGTTGTTG
RAP with XhoI for pWIZ	CTCCTCGAGAATCAGATTGTAGGTGTCCAAGTC
Dm-alpha F	TTGAATTCAGTGCCGTGTACAACACGAAG
Dm-alpha R	CTCCTCGAGTTAGAATTGATCCGTCAACAGATC
Dm Ced-6 S444-stop	TAACAGCAGTGGCTTCTAGAGCGAGCTGAACATAACGCCG
GC - Dm Ced-6 S444-stop	CGGCGTTATGTTCAGCTCGCTCTAGAAGCCACTGCTGTTA
Dm Ced-6 K251- stop	CACCGAAAAGACGAATCTGAGCTAGGCGCAGCAACAGATTAATC
GC - Dm Ced-6 K251- stop	GATTAATCTGTTGCTGCGCCTAGCTCAGATTCGTCTTTTCGGTG
Dm Ced6 L235-stop	GGCCAGGCCTTCGATTAGGCCTACAGAAAGTAC
GC - Dm Ced6 L235-stop	GTACTTTCTGTAGGCCTAATCGAAGGCCTGGCC
Dm Ced6 DPF-APA	ACAGATCTATTCGGTTCGGCTCCTGCCGAATTGAACAATGGACCG
GC - Dm Ced6 DPF-APA	CGGTCCATTGTTCAATTCGGCAGGAGCCGAACCGAATAGATCTGT
Dm Ced6 LID-AAA	GGCAAGCTGGATGATGATAAGCTGGCAGCCGCCACCAATTCCACCACAGCG
GC - Dm Ced6 LID-AAA	CGCTGTGGTGGAATTGGTGGCGGCTGCCAGCTTATCATCATCCAGCTTGCC
5'GFP-EcoRI	TTTGAATTCGCCACCATGGTGAGCAAG
Ced-6 PTB reverse	CTCCTCGAGCTAGTTTACGGTTTGTTGCAGATG
Ced-6 C1 Forward	TTTGAATTCGTTTACAAGGAGCGACTGCG
CED6 Xho1 3'	CTCCTCGAGCTACTTTCGCAGGGGATCC
Add stop and XhoI to 3'	TTTCTCGAGTTACTTGTACAGCTCGTCCATGCC

eGFP	
T7-RAP-Forward	GCGTAATACGACTCACTATAGGGAGACTTAAGCCCTACAAGAAATTCCAC
T7-RAP-Rev	GTTTTAATGGACTCCAGTTCTTTAAC
RFP F for RAP	TTTGAATTCATGGTGAGCAAGGGCGAGG
RFP R for RAP	GCGAATTCGGTACCCTTGTACAGCTCGTCCATGC
Skittles-F	CCGAATGGCATATTCCTGGAG
Skittles -R	GCGTAATACGACTCACTATAGGGAGAGCGTCCTGGAAGCGCTTGGCG
Yolkless FxNP to AxNP	GGATCTCAACATTAACATGGCTTTCCAGAATCCGCTGGCGACG
GC - Yolkless FxNP to AxNP	CGTCGCCAGCGGATTCTGGAAAGCCATGTTAATGTTGAGATCC
Dm Yolkless FxN to AxN	CTCAACATTAACATGCATGCCCAGAATCCGCTGGCGACG
GC - Dm Yolkless FxN to AxN	CGTCGCCAGCGGATTCTGGGCATGCATGTTAATGTTGAG
Dm Yolkless FxNP to FxAA	GGATCTCAACATTAACATGCATTTCCAGGCTGCGCTGGCGACGCTC
GC - Dm Yolkless FxNP to FxAA	GAGCGTCGCCAGCGCAGCCTGGAAATGCATGTTAATGTTGAGATCC
Dm Yolkless PxA to PxY	GCATTTCCAGAATCCGCTGTATACGCTCGGCGGCACC
GC - Dm Yolkless PxA to PxY	GGTGCCGCCGAGCGTATACAGCGGATTCTGGAAATGC
3.5kb RAP-F1	CGGGGTACCGGAGAGGGTGCAGGATGCTCGGG
3.2kb RAP-F2	CGGGGTACCGACATCGATAGGATGATAGAGGTCG
RAP-F3	CGGGGTACCCACAAGCCAGCGCCACAAGGGAC
RAP-Rev	GCTCTAGACCACGCCACACCACACCACACCA
Aa alpha insitu F	CAAACGGCGTTGCAGAATTTCG
Aa AP-2 alpha rev	GCGTAATACGACTCACTATAGGGAGAGTTCTCTAAACTGGAACGAATCG
RAP5'with NotI	TTTGCGGCCGCGCCACCATGGTTAGGTCAGCTCTCGTTG

RAP3' with XbaI	TAGTCTAGATTAAAGCTCGGTGTGCTTGAAG
Ced6-F	CGACACCAATTCCACCACAG
Ced-6-Rev	GCGTAATACGACTCACTATAGGGAGGATCCAAACTCTCCAGCG
DmCed6 369 stop	GCAATAGCCAAAAGATGGACTAGCTGCTGCTGAACTCG
GC - DmCed6 369 stop	CGAGTTCAGCAGCAGCTAGTCCATCTTTTGGCTATTGC
Dm Ced6 468 stop	CCCAGGCGCCTAAACTAGGTGACAACGGGCAAC
GC - Dm Ced6 468 stop	GTTGCCCGTTGTCACCTAGTTTAGGCGCCTGGG
Ced6 376 start	GAGCTGCTGCTGAACTCGGAATTCGATAGTGATTTCGATCCG
GC - Ced6 376 start	CGGATCGAAATCACTATCGAATTCCGAGTTCAGCAGCAGCTC
Dm Alpha W842A	GCGGAATCATTCTTTGCACGAGCGAAGAACCTTAGCGGTG
GC - Dm Alpha W842A	CACCGCTAAGGTTCTTCGCTCGTGCAAAGAATGATTCCGC
Dm Alpha R918A	CAGGCTCAGATGTTCGCACTGACAGTTCGGGCAAGC
GC - Dm Alpha R918A	GCTTGCCCGAACTGTCAGTGCGAACATCTGAGCCTG
DmCed6-401+	TTTGAATTCATGTTCGGCTTCGAGCCGG
Ced6-370F	TTTGAATTCCTGCTGAACTCGGATTCCG
Ced6-430F	TTTGAATTCCTGATAACGAGCAATAACAACAG
Ced6QC430stop	GCTGCAAAATAATAACAGCAGCTGACTGATAACGAGCAATAACAAC
GC - Ced6QC430stop	GTTGTTATTGCTCGTTATCAGTCAGCTGCTGTTATTATTTTGCAGC
Ced6QC500stop	CCGAACATTTTCAAGCAAAATTAGCTTAACCTTGACGACTTCTCG
GC - Ced6QC500stop	CGAGAAGTCGTCAAGGTTAAGCTAATTTTGCTTGAAAATGTTCGG
Ced6-C1 F for pet28a	TTTCATATGGTTTACAAGGAGCGACTGCG

DmYP1-Forward	GGAAGATCTGCCACCATGGAACCCCATGAGAGTGCTG
DmYP1-Reverse	TCCCCGCGGCTGGTAGTCCTTGCTGTCC
RFP-Forward for pUAST-RFP N1	GCTCTAGAATGGTGAGCAAGGGCGAGG
RFP-Reverse for pUAST-RFP-N1	GCTCTAGACGCTAGCTTACTTGTACAGCTCGTCCATGC
RFP-Forward for pUAST-RFP-C1	TTTGAATTCCACCATGGTGAGCAAGGGCGAGG
RFP-Reverse for pUAST-RFP-C1	GCGAATTCCTTGTACAGCTCGTCCATGC
Dm-Dab-F	TTTGAATTCATGGTCAAGTCCCTGGTGG
Dm-Dab (1-200)	GCGCGGCCGCTCACGACGACAGGGAGGCCAG
Dm Dab (1-500)	GCGCGGCCGCTCAGCTGAGCTCCTTGAGCAGC
Dm Dab-ISH F-New	CCAGTTGCAATTCCACGCCG
Dm-Dab-ISH-New R	GCGTAATACGACTCACTATAGGGAGACACTGAAATTTGCCGCCCTC
Ced6-DLF-ALA	CTGAATGGCAACACAGCTCTAGCCGGTTCGGCTCCTGCCG
GC - Ced6-DLF-ALA	CGGCAGGAGCCGAACCGGCTAGAGCTGTGTTGCCATTCAG
Dm Dab2145F	TTTGAATTCATGGAAACACCGCCGACC
Dm Dab2244R	GCCTCGAGTCAAAACTCGTCATCCTCGAAGG
Dm-Dab-AD-F	TTTCATATGGTCAAGTCCCTGGTGG
DmDab-AD-R	GCGAATTCTCAGCTGAGCTCCTTGAGCAGC
Ced6-F-NdeI	TTTCATATGCCTTATCAGCCAGCCAAC
Dm-YL F1878Y	GCAGCAACGACACATACACCACCACCAGCGC
GC - Dm-YL F1878Y	GCGCTGGTGGTGGTGTATGTGTCGTTGCTGC

Dm YL LL1918AA	CCGATGGCTCAGGAATTGGCTGCCGAGAGCCCCAGC
GC - Dm YL LL1918AA	GCTGGGGCTCTCGGCAGCCAATTCCTGAGCCATCGG
Ced-6 without stop XhoI	GCCTCGAGCTTTCGCAGGGGATCCAAACTC
Ced-6 without stop KpnI	CGCGGTACCGCTTTCGCAGGGGATCCAAACTC
Ced6242stop	GGCCTACAGAAAGTACATGTAGAGCACCGAAAAGACGAATCTG
GC - Ced6242stop	CAGATTCGTCTTTTCGGTGCTCTACATGTACTTTCTGTAGGCC
Drpr f	TTTCATATGCGCCGACGTGTGTCCAAT
Drpr R	GCGAATTCCTACTTTAGAAATTTCGGACTG
Dm-Yolkless-both-RT-F	CGGATCTCAACATTAACATTAACATGC
Dm-Yolkless-SI-R	CATATCTTCCACGGCCATCTG
Dm-Yolkless-LI-R	CATTCGCATCATTGCCTGCG
Ced6-S164T	CCGCTGTACAACATTACGTATTGTGCGGACGAGAAG
GC - Ced6-S164T	CTTCTCGTCCGCACAATACGTAATGTTGTACAGCGG
Ced6-F233V	CTGACCATTGGCCAGGCCGTCGATTTGGCCTACAG
GC - Ced6-F233V	CTGTAGGCCAAATCGACGGCCTGGCCAATGGTCAG
Ced6-S164Y	CAGTTTCCGCTGTACAACATTTATTATTGTGCGGACGAGAAGGG
GC - Ced6-S164Y	CCCTTCTCGTCCGCACAATAATAAATGTTGTACAGCGGAAACTG
Ced6-E301Stop	GGAAGCGCCCACCACATAACCCCAGAATGGAATCG
GC - Ced6-E301Stop	CGATTCCATTCTGGGGTTATGTGGTGGGCGCTTCC
CED6-(475-517)-F	TTTGAATTCGGCCTGAATGGCAACACAG
CED6-(492-517)-F	TTTGAATTCGGACCGAACATTTTCAAGC
Hs GULP CT	GTATTTTGTCTCGACTCGTTAGACCCTCTGCGCTGACTCGAGCGGCCG

QC #1	
GC - Hs GULP CT QC #1	CGGCCGCTCGAGTCAGCGCAGAGGGTCTAACGAGTCGAGACAAAATAC
Hs GULP CT QC #2	ATGGGACTAACTCTTGAAGGCGACGACTTTTCTCTCGACTCGTTAGACCCTC TG
GC - Hs GULP CT QC #2	CAGAGGGTCTAACGAGTCGAGAGAAAAGTCGTCGCCTTCAAGAGTTAGTCC CAT
Hs GULP CT QC #1A	TTTTGTCTCGACCCGTTAGACCCTCTGCGCTGACTCGAGCGGCCGC
GC - Hs GULP CT QC #1A	GCGGCCGCTCGAGTCAGCGCAGAGGGTCTAACGGGTCGAGACAAAA

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